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CORNELL UNIVERSITY AGRICULTURAL EXPERIMENT STATION

OF THE COLLEGE OF AGRICULTURE

PHYSIOLOGICAL STUDIES OF BACILLUS RADICICOLA OF CANADA FIELD PEA

By MARTIN J. PRUCHA

FORMERLY ASSISTANT PROFESSOR OF PLANT PHYSIOLOGY
AT CORNELL UNIVERSITY

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PHYSIOLOGICAL STUDIES OF BACILLUS RADICICOLA OF CANADA FIELD PEA

PHYSIOLOGICAL STUDIES OF BACILLUS RADICICOLA OF CANADA FIELD PEA[1]

MARTIN J. PRUCHA

INTRODUCTION

Ever since the discovery that the formation of nodules on the roots of legumes is associated with a definite microorganism, there have appeared on the market pure cultures of this organism for the artificial inoculation of leguminous plants. Nobbe and Hiltner (1897)[2] first caused to be placed on the market a pure culture called nitragin,[3] in which the medium employed was gelatin. The efficiency of the culture was low, and Nobbe and Hiltner explained this by ascribing to the culture a loss of infecting power due to the medium used. Moore (1905) stated, as a result of his work, that the virulence of the organism was impaired when it was propagated on nitrogenous media, and consequently he used a nitrogen-free medium.

The same opinion has been held by various other investigators, and is observed by all firms and institutions that distribute pure cultures of the legume organism. Despite the fact that media low in nitrogen have been employed, the results with pure cultures have not always been satisfactory, and Kellerman (1912) has stated that the pure cultures have not been as efficient as field soil. It would seem, then, that the conditions of isolation and cultivation of the legume organism must influence the organism, or that possibly the soil conditions would exert some influence on the efficiency of the organism to effect inoculation or would have an influence on the plant to resist infection.

SCOPE OF THE INVESTIGATION

With these ideas in mind, the investigation herewith reported was undertaken. It is concerned with the following: (1) Isolation and identification of the organism causing nodule development on the roots of Canada field pea; (2) A study of the influence of various factors on nodule

[1] Laboratory of Plant Physiology, Contribution No. 15.

[2] Dates in parenthesis refer to bibliography, page 79.

[3] This is not the nitragin now on the market.

development in Canada field pea when the plant is grown in water or soil cultures; (3) A study of the influence of various environmental conditions on the infecting power of the organism.

METHOD OF INVESTIGATION

Organism

Bacillus radicicola from Canada field pea was used for all the experiments except number 11, for which the organism from alfalfa was used. The organism was isolated as described on page 14. The stock culture was kept in the laboratory on agar slopes of medium 335, and was exposed to diffused light during the entire investigation. It was transferred at irregular intervals of time varying from one to three months.

Media

The various kinds of media used are referred to in the text by their laboratory numbers. In table 1 are given the laboratory numbers and the composition of the principal media employed. In addition to these a number of other media were used, and these are described subsequently. All the media were prepared according to the recommendations on the Descriptive Chart of the Society of American Bacteriologists.

Sterilization of utensils

Strict precautions were observed in sterilizing all the utensils. Glassware was sterilized for two hours at from 150° to 170° C. Scalpels and similar instruments, which resist high temperature, were heated in the flame. For work requiring air free from dust, a room was used into which a stream of steam was allowed to flow until the air was saturated; after condensation of the steam, the air in the room was conspicuously free from any dust.

Sterilization of seed

The experiments in Part III required seed free from *B. radicicola*. It was necessary, therefore, to sterilize the seed. In experiments 10 and 11, 95-per-cent alcohol was used, the seed being treated for thirty seconds. In experiments 12 and 13 a solution of bleaching powder was used. The author is indebted for this method to Professor J. K. Wilson, of Cornell University. The solution is prepared by mixing 8 grams of bleaching

TABLE 1. LABORATORY NUMBERS, COMPOSITION, AND REACTION OF MEDIA

Laboratory number	Composition	Reaction
Medium 101	0.2 gram $MgSO_4$ 1 gram KH_2PO_4 0.3 gram $Ca(NO_3)_2$ 0.01 gram $FeCl_3$ 1000 cc. distilled water	Not changed
Medium 300	15 grams agar 3 grams Liebig's beef extract 10 grams Witte's peptone 1000 cc. distilled water	+1, Fuller's scale
Medium 310	Same as 300, plus 2 per cent dextrose	Neutral
Medium 331	15 grams agar 10 grams cane sugar 1 gram KH_2PO_4 0.2 gram $MgSO_4$	Not changed
Medium 334	15 grams agar 0.2 gram K_2HPO_4 0.2 gram $MgSO_4$ 0.2 gram NaCl 0.2 gram $CaSO_4$ 0.2 gram $CaCO_3$ 1000 cc. tap water	Not changed
Medium 335	Same as 334, plus 20 grams cane sugar	Not changed
Medium 337	Same as 335, plus 10 grams Witte's peptone	Not changed
Medium 400	15 grams agar 3 grams Liebig's beef extract 20 grams Witte's peptone 1000 cc. distilled water	+1, Fuller's scale
Crone's solution	1 gram KNO_3 0.5 gram $Fe_3(PO_4)_2$ 0.25 gram $CaSO_4$ (gypsum) 2000 cc. distilled water	Not changed
Pfeffer's solution	4 grams $Ca(NO_3)_2$ 1 gram KNO_3 1 gram $MgSO_4$ 0.5 gram KCl 0.06 gram $FeCl_3$ 6000 cc. distilled water	Not changed

powder with 140 cubic centimeters of water; after standing for a few hours, the clear liquid is decanted and is then ready for use. Seed was kept for three hours in this solution. According to Professor Wilson's results, seed treated in this way is completely sterilized. This method of seed sterilization will later be published in detail.

That the methods of seed sterilization employed were effective was proved by a large number of experiments, and particularly by experiments with soy beans. The organism producing nodules in soy bean is not present in the soil of this region, and in all the experiments made not a single nodule developed in the cultures in which sterilized seed was used unless the plants were subsequently inoculated. Unsterilized seed occasionally produced plants with nodules.

Sterilization of media and soil

All the media used for pure cultures, if in test tubes of small volume, were sterilized in an autoclave for fifteen minutes at 120° C.

In experiments 10, 11, 12, and 13 it was necessary to sterilize the soil in which the plants were grown. Three-inch flowerpots and glass tumblers, each containing about 300 grams of soil, were used for this purpose. These were sterilized for three hours at 120° C., in a large canner's retort. This retort was found very useful, since several hundred of the flowerpots could be sterilized at one time.

Method of growing the plants

In experiments 1, 2, 3, 4, 5, and 6, the plants were grown in water cultures; in experiments 7, 8, 9, 10, 11, 12, and 13 they were grown in soil.

For the water cultures glass vessels were employed. The vessels were filled with the nutrient solution and the opening was covered with paraffin paper. The seed was then germinated in a moist chamber, and when the radicle was about three centimeters long it was inserted into the solution through a small hole in the paraffin paper, allowing the cotyledons to rest on top of the paper.

For the soil cultures flowerpots and glass tumblers were used. These were filled with sandy soil, were covered with paper, and, for experiments 10, 11, 12, and 13, were sterilized. The seed was planted directly in the soil, the paper covers being kept on until the seedlings began to push them off.

Distilled water was used for watering the plants in experiments 1, 2, 3, 4, 5, 6, 7, 8, and 9. In experiments 10, 11, 12, and 13, boiled tap water was used.

The plants in all the experiments were grown in the greenhouse. When special precautions were necessary to guard against contamination during the growing period, the plants were kept in an especially constructed culture room. For this purpose a part of the greenhouse was set off by a partition. Cracks in the walls and around the panes of glass were filled with plaster of paris. For ventilation, two panes of glass were replaced by a special frame fitted with a layer of cotton held between two pieces of cheesecloth.

Inoculations

Inoculations were made at the time of planting the seeds or within a day or two following. The culture of the organism to be used for inoculation was introduced into sterile water, and the infusion was then added to the soil or the water culture in which the plants were grown. In some experiments quantitative inoculation was made, in which a specific amount of the infusion was added.

Examination of plants for nodules

Nodules usually appeared in about two weeks. The plants were examined at the end of three weeks. The roots were washed and the nodules were counted, and a note was made of the size and the place of attachment of the nodules. Since the soil in the vicinity of Ithaca is well inoculated with the Canada field pea organism, plants kept longer than three weeks were subject to some contamination.

Special method for growing plants under sterile conditions

For certain experiments it was necessary to maintain absolutely sterile conditions throughout the period of experiment. The method employed was as follows: A large glass cylinder, 65 centimeters high and 10 centimeters in diameter, was used as a growth chamber. In the bottom of the cylinder a few pieces of broken flowerpot were placed and were just covered with water, and on the top of these was set a four-inch pot, filled with a sandy soil. The cylinder was plugged with cotton, through which was passed a glass tube 7 millimeters in diameter and 65 centimeters long, the lower end resting on the surface of the soil in the pot

and the upper end protruding above the cotton plug. The tube was plugged at its upper end with cotton. The whole was sterilized in the autoclave for five hours at 15 pounds pressure.

The seeds were sterilized by the bleaching-powder method. The sterilized seed were dropped into the pots through the glass tube, and by manipulation of the tube they were buried in the soil. The soil of the pots was inoculated by introducing through the tube a few cubic centimeters of water containing the nodule-forming organisms.

PART I. ISOLATION AND IDENTIFICATION OF THE ORGANISM

ISOLATION OF BACILLUS RADICICOLA

On October 10, 1910, a field pea plant 30 centimeters high, with a great abundance of nodules, was procured. The whole plant was washed thoroughly in running water. One of the nodules, of firm consistency, was selected and cut off in such a way as to leave about 3 centimeters of the root on each side of the nodule; cut off in this way the nodule is more easily manipulated. The nodule was then disinfected in a solution of one part of formalin to forty parts of water, for five minutes. Four petri dishes were prepared, each containing a few drops of sterile water. The nodule, after being disinfected, was washed in sterile water, placed on a filter paper, and cut open, and with a pointed scalpel a part of the central tissue was removed and placed in petri dish 1. This nodule tissue was crushed and mixed with the water. Three loopfuls of this infusion was transferred from plate 1 to plate 2, three loopfuls from plate 2 to plate 3, and three loopfuls from plate 3 to plate 4. Ten cubic centimeters of medium 331 were then poured into each petri dish, and after sufficient agitation to effect equal distribution of the organisms the plates were allowed to incubate at 20° C. In three days a few colonies characteristic of the nodule organism became visible on plate 1; on the other plates plenty of colonies were present, but were visible only by the microscope. In ten days the small colonies became large enough to be conveniently transferred.

As far as could be ascertained from the general appearance of the colonies, all the plates contained only one organism. The large colonies that developed on plate 1 appeared to be giant colonies, having started from small pieces of the nodule tissue in which a large number of the organisms were held. In order to obtain a pure culture of this organism, one of the

small colonies was introduced into 5 cubic centimeters of sterile water in a test tube, and from this infusion a number of petri dishes of different dilutions were again prepared. From one of the colonies that developed on these petri dishes a transfer was made on agar slope with medium 335. This culture was used in all the experiments except experiment 11. The stock culture was kept on a shelf in the laboratory, and consequently was exposed for nearly three years to diffused light and to the ordinary variations of temperature and other atmospheric changes. The transferring of the stock culture was made at irregular intervals of time varying from one to three months, and the cultures were kept in test tubes on agar slopes of medium 335.

IDENTIFICATION OF THE ORGANISM

In order to be certain that the organism isolated was the causal organism of the nodules on Canada field peas, the following procedure was followed: (1) Canada field peas were grown under sterile conditions and were inoculated with this organism; (2) from one of the nodules that developed under sterile conditions a culture, No. 2, was isolated by the same method as described above; (3) the original culture and the culture No. 2 were again tested as to their ability to cause the development of nodules on Canada field peas under sterile conditions; (4) the two cultures were compared in the laboratory with respect to their morphological characters and physiological activities.

Ten Canada field peas were grown under sterile conditions according to the method described on page 13, one plant in each of ten glass cylinders. Five of the plants were inoculated with the above organism, and five were left without inoculation, as controls. At the end of six weeks the plants were examined. They had made a fair growth, having reached a height of 60 centimeters. They were spindling, however, the leaves were small, and the root system was very poorly developed. All the inoculated plants had nodules on their roots, while the controls were free from any nodules. Each plant and the soil in which it was grown were examined for contamination. One of the controls was found to be contaminated with a mold, and one of the inoculated plants was contaminated with a yellow organism. Four of the controls were sterile, and four of the inoculated plants were found to be sterile with respect to organisms other than *B. radicicola.*

A pure culture of the organism was isolated from a nodule found on one of the plants grown under sterile conditions. This organism was again tested as to its ability to produce nodules on Canada field peas. Fifteen Canada field pea plants were grown under sterile conditions again, in a similar manner to that described above. Five were inoculated with this organism, five were inoculated with the original organism, and five were left as controls. Again all the plants inoculated with both the cultures developed nodules on their roots, while the controls had none. The organism that was isolated on October 10, 1910, therefore, was the causal organism of nodules on Canada field pea plants, and as far as could be determined by laboratory methods it was also a pure culture.

The two cultures of the organism — the one isolated originally, and the other isolated from a nodule of a plant grown under sterile conditions — were studied in the laboratory with respect to their morphology and their physiological activities. An exhaustive study of this phase was not undertaken, the study being carried only far enough to show whether the two cultures were the same organism. The study consisted in propagating the two cultures on various media and in comparing and describing their cultural and biochemical features. The descriptions were recorded on the Descriptive Chart adopted for such use by the Society of American Bacteriologists (1907).

Morphology of the organism

Bacillus radicicola of Canada field pea produces no spores when propagated on the artificial media in the laboratory. In a young culture on artificial media, the organism is in the form of small rods about one micron long. In this form it is able to multiply by fission, like other bacteria. Under certain conditions — for example, with the addition of certain nutrients, such as sugar, to the media — some of these small rods develop into large cells, which are generally called bacteroids. Some of the bacteroids assume the characteristic X and Y forms, the same as are found in the nodules. The development of the bacteroids seems to be largely a matter of nutrition, and the bacteroids are not a degenerate form, but a normal form, of the organism.

In a culture twenty-four hours old on an agar slope the organism is very motile. As the agar-slope culture gets older, fewer and fewer of the organisms show motility, until in a culture about two weeks old no motility

is detected. Motility seems to be influenced by environmental conditions. This point has not been studied extensively, beyond the observation that on nitrogenous media the motility is lost sooner than on nitrogen-free media.

Since the generic name of the organism depends on the presence and the place of attachment of flagella, and since there has been so much uncertainty on this point, an effort was made to demonstrate the number and the arrangement of the flagella. Beyerinck (1890) was the first who claimed to have isolated the organism in a pure culture. He described it as having one polar flagellum, and named it *Bacillus radicicola*. In 1905, Moore, agreeing with Beyerinck as to the number of flagella and wishing to conform to Migula's classification, changed the generic name from *Bacillus* to *Pseudomonas*. Edwards and Barlow (1909) found only one long, whiplike flagellum, thus agreeing with Beyerinck and Moore. De' Rossi (1907) was the first investigator who found the organism of *Vicia faba* to have about eight flagella with a peritrichic arrangement. Zipfel (1912) agreed with de' Rossi, stating that the organism has numerous flagella. In 1912 Kellerman, using a special method, also succeeded in staining the organism of several leguminous plants. He likewise found the organism to have several flagella peritrichically arranged.

In this investigation the following method was used for staining the organism of Canada field pea for flagella:

An agar slope culture twenty hours old, on medium 335 at 24° C., was very carefully transferred into 3 cubic centimeters of sterile distilled water in a test tube. This was allowed to stand for about four hours at a constant temperature, without being shaken or disturbed in any way. A drop of this infusion was placed on a cover glass and allowed to dry at room temperature, and when dry it was stained.

It was essential that the cover glasses should be clean. To this end they were treated with a cleaning solution of potassium bichromate and sulfuric acid, washed in water, placed in alcohol, and finally dried with a piece of cheesecloth which had been treated with ether in order to get rid of any fats. The cover glasses were then placed in a petri dish and baked in the oven for three hours at 200° C. If cover glasses are treated in this way a large amount of difficulty in the staining of flagella is avoided.

Pitfield's mordant as modified by Muir, and carbol fuchsin, were used for staining. The mordant has the following composition:

	Cubic centimeters
Tannic acid, 10 per cent aqueous solution	10
Corrosive sublimate, saturated aqueous solution	5
Alum, saturated aqueous solution	5
Carbol fuchsin	5

The mordant was applied for six minutes and the preparation was then very thoroughly washed with water. Carbol fuchsin was then applied for nine minutes.

It was found that the organism from Canada field pea has peritrichic arrangement of flagella. The largest number of flagella observed was six, arising from any part of the organism, and the indications were that the organism may have an even larger number. According to Migula's classification the organism is *Bacillus*.

Cultural and biochemical features

The surface colonies in a petri dish on agar medium 335 are colorless, watery, and very viscous. When ten days old, at 20° C., they are about 3 millimeters in diameter, although occasionally colonies 10 millimeters in diameter may develop. The colonies under the surface are invariably of spindle shape. Under the 16-millimeter objective the microscopic structure appears granular.

On agar slope with peptone and beef extract, the growth is watery, scanty, and colorless at first; after long standing it becomes brownish. In standard gelatin stab the growth becomes brownish. On agar slope with medium 335 to which 0.5 per cent of potassium or calcium nitrate had been added, the growth becomes opaque and iridescent. In all the standard media free from sugar the growth is scanty. In the presence of dextrose, saccharose, maltose, or glycerin, much more abundant growth takes place. Lactose and galactose increase the growth only slightly, while the addition of levulose tends to inhibit it. Two per cent of levulose added to media entirely inhibits the growth. Whether this is due to this sugar or to some impurity in it was not determined.

The organism does not produce any indol, hydrogen sulfite, or ammonia. It does not reduce nitrates and does not liquefy a 12-per-cent gelatin stab at 20° C., but when grown in milk it partly digests the casein without curdling the milk. It does not produce any gas from the sugars in fer-

mentation tubes. From dextrose, maltose, and saccharose it produces a slight amount of some acid. Neutral litmus milk becomes alkaline after the organism has grown in it for about two weeks.

The organism of Canada field pea does not have any strikingly characteristic colony features by which it can be distinguished from other microorganisms. The group number of the organism, according to the chart of the Society of American Bacteriologists, was found to be B. 222.2322033.

PART II. INFLUENCE OF CERTAIN FACTORS ON NODULE PRODUCTION

The literature on the general subject of legume inoculation is very extensive; yet knowledge concerning the factors that influence nodule production is suprisingly meager. For the most part the investigations on this point are incidental and fragmentary. It has been shown by various investigators, however, that the nature of the medium in which the plant is grown has an influence on the production of nodules.

Rautenberg and Kühn (1864) grew *Vicia faba* in various nutrient solutions, and incidentally observed that in the nitrogen-free solution the beans developed an abundance of nodules, while in the solutions containing nitrogen no nodules were produced.

Hugo de Vries (1877) made a similar observation. He grew red clover in nutrient solutions, and the plants developed a large number of nodules in the nitrate-free solution but only a few or no nodules in the solution containing nitrates.

Vines (1888–1889) tested the influence of potassium nitrate on nodule development on *Vicia faba* grown in nutrient solutions and also in the soil. His experiments showed that potassium nitrate tends to inhibit nodule development, both in soil and water cultures.

Frank (1889) grew lupines and peas in humus soil and in humus-free soil. He found that the plants grown in the humus-free soil developed an abundance of nodules, and those grown in the soil rich in humus had no nodules. He offered the following explanation for this: "The lupines, and also the peas, obtain the same benefit from the nodule fungus as they do from the humus. Where humus is present, the plants prefer to obtain the nourishment from the humus and no nodules are developed; where humus is wanting, however, the nodule fungus infects the plants."[4]

[4] Translation from the original German.

Hiltner (1900) showed that the addition of potassium nitrate to the nutrient solution in which legumes are grown has an injurious influence on nodule development, and he thinks this is due to the fact that the formation of bacteroids in the small nodules is hastened by the presence of the nitrate. He considers the bacteroids as degenerate and inactive forms of the nodule-forming organism.

A somewhat more extensive investigation of this subject was undertaken by Nobbe and Richter (1902). They attempted to determine the influence of potassium nitrate and of humous substances on the fixation of nitrogen by soy beans. They grew the plants in flowerpots in a rich soil and in a poor soil. For rich soil they used garden soil. The poor soil was prepared by mixing 4000 grams of sand and 2500 grams of garden soil. Potassium nitrate was added to the poor soil in the proportions of 500 and 1000 milligrams to 6500 grams of the soil. When the plants were harvested the total amount of dry substance and of nitrogenous matter was determined in each plant. The results of this experiment show that the function of the nodules for nitrogen fixation is injured to a high degree by the presence of potassium nitrate. The influence of humous substances is similar, but not so great.

The observation made by Frank, by Nobbe and Richter, and by others — namely, that soil rich in humous substances has a deleterious effect on nodule development — was confirmed also by Moore (1905). He grew soy beans in rich nitrogenous soil, in poor clay soil, and in poor sandy soil. Very few nodules developed on the soy beans grown in rich soil, while in the poor clay soil and in the poor sandy soil the plants developed an abundance of nodules.

The development of nodules may also be affected by other agencies. Gain (1893) attempted to determine the influence of moisture on nodule development on *Pisum sativum*, two varieties of *Lupinus albus*, and *Faba vulgaris*. He grew the plants in a field located in a region where rain was very scarce during the early part of the summer. One-half of the plat planted with each legume was watered artificially, and the other half was exposed to drought. Examination of the plants showed that the plants watered artificially had five and one-half times as many nodules as those not watered.

Marchal (1901) determined the influence of fifteen different nutritive mineral salts on nodule development on peas grown in Sachs' nutrient

solution. He concluded from his experiments that nodule development is inhibited by the addition of the following nutrient salts in the given concentrations:

Alkaline nitrates, concentration 1 to 10,000
Ammonium salts, concentration 1 to 2,000
Potassium salts, concentration 1 to 300
Sodium salts, concentration 1 to 200

The influence of phosphates was variable, and calcium and magnesium salts stimulated nodule development. Marchal was of the opinion that the variation of the osmotic pressure, due to the presence of the different salts, may be the cause of this phenomenon.

Moore (1905) states that the addition of 1 per cent of sodium and potassium salts often entirely inhibits the formation of nodules, and smaller quantities considerably reduce their formation. The addition of calcium and magnesium salts, on the other hand, greatly favors nodule formation. Moore states further that this is not true with all the legumes, since the addition of calcium and magnesium carbonates is injurious to the formation of nodules on lupines and other plants adapted to acid soil.

In the following experiments the influence of various factors on nodule production has been investigated. The factors studied are light and darkness, aëration, moisture, various concentrations of nutrient solutions, and a considerable number of chemical substances.

EXPERIMENT 1

INFLUENCE OF AËRATION IN LIGHT AND IN DARKNESS

In this experiment Canada field peas were grown in an aqueous soil extract. The extract was prepared by taking one part of soil and four parts of water, by weight. The mixture was allowed to stand for two hours, and the liquid was then decanted and filtered. Twelve Erlenmeyer flasks of 300 cubic centimeters capacity were filled, and one pea was planted in each flask. Six of the flasks were covered with black paper and the other six were exposed to diffused light. Three flasks n each of the two series were aërated by passing a current of air through the liquid during the entire experiment. All the plants were inoculated with

a pure culture of *Bacillus radicicola*. After twenty-four days the plants were examined for nodules.

Results

All the plants developed plenty of nodules. The inoculation with the pure culture had no apparent effect on the number of nodules. The soil extract was not sterilized and apparently had plenty of the organisms. The plants whose roots were kept in darkness had a greater abundance of nodules than those whose roots were exposed to light. The aëration as supplied in this experiment had no stimulative effect on either the number or the size of the nodules.

EXPERIMENT 2

INFLUENCE OF SOME NUTRIENT SOLUTIONS IN LIGHT AND IN DARKNESS

Ninety Erlenmeyer flasks of 300 cubic centimeters capacity were divided into five series, with eighteen flasks in each series. These flasks were filled with the following solutions, respectively: series 1, with medium 101; series 2, with Crone's solution; series 3, with Pfeffer's solution; series 4, with tap water; series 5, with soil extract (the same as was used n experiment 1). Six flasks from each series, three covered with black paper and three not covered, were inoculated with a pure culture of *Bacillus radicicola*. A second group of six flasks from each series were prepared in the same manner, but each flask was inoculated with 5 cubic centimeters of soil extract. A third group of six flasks from each series were prepared in the same manner but were not inoculated. One plant was grown in each flask. The water of transpiration was replaced each week. At the end of four weeks the plants were examined for nodules. The results are given in table 2.

Results

Not all the plants that were inoculated developed nodules. A few nodules developed on the plants grown in medium 101, in Crone's solution, and in Pfeffer's solution. In the soil extract all the plants developed nodules, although the plants grew better in Crone's solution and in Pfeffer's solution. In the tap water no nodules developed. More nodules developed on the roots kept in darkness than on those exposed to the light.

TABLE 2. INFLUENCE OF SOME NUTRIENT SOLUTIONS ON NODULE DEVELOPMENT

Culture solution*	Treatment	Exposure	Number of nodules		
			Plant 1	Plant 2	Plant 3
Medium 101	Inoculated with *B. radicicola*	Light	None	None	None
		Dark	None	None	None
	Inoculated with soil extract.	Light	None	None	None
		Dark	None	None	Few
	Not inoculated	Light	None	None	None
		Dark	None	None	None
Crone's solution	Inoculated with *B. radicicola*	Light	None	None	None
		Dark	Few	Few	Few
	Inoculated with soil extract.	Light	None	None	None
		Dark	Few	Few	Few
	Not inoculated	Light	None	None	None
		Dark	None	None	None
Pfeffer's solution	Inoculated with *B. radicicola*	Light	None	None	None
		Dark	None	None	Few
	Inoculated with soil extract.	Light	None	Few	Few
		Dark	None	Few	Few
	Not inoculated	Light	None	None	None
		Dark	None	None	None
Tap water	Inoculated with *B. radicicola*	Light	None	None	None
		Dark	None	None	None
	Inoculated with soil extract.	Light	None	None	None
		Dark	None	None	None
	Not inoculated	Light	None	None	None
		Dark	None	None	None
Soil extract	Inoculated with *B. radicicola*	Light	Present	Present	Present
		Dark	Present	Present	Present
	Not inoculated	Light	Present	Present	Present
		Dark	Present	Present	Present

* See page 11.

EXPERIMENT 3

INFLUENCE OF POTASSIUM NITRATE IN LIGHT AND IN DARKNESS

In this experiment twelve glass cylinders, each of 5 liters capacity and 50 centimeters in height, were used. Six of these were filled with Crone's full nutrient solution, and the other six were filled with the same solution except that potassium chloride was substituted in place of potassium nitrate. Five plants were grown in each cylinder. The experiment was arranged in the following manner:

Series 1. Three of the cylinders filled with Crone's full nutrient solution were covered with black paper. Two of these were inoculated, and one was not inoculated.

Series 2. The other three cylinders filled with Crone's full nutrient solution were treated as was series 1, but were not covered with black paper.

Series 3. Three of the cylinders filled with Crone's solution in which potassium nitrate was replaced by potassium chloride, were covered with black paper. Two of these were inoculated, and one was not inoculated.

Series 4. The remaining three cylinders, with the same solution as was used for series 3, were treated as was series 3 but were not covered with black paper.

Results

When the plants were three weeks old, those grown in the solution with nitrate looked green and healthy, while those grown in the solution without nitrate were turning yellow and the lower leaves were dropping off. No difference in appearance was observed between the inoculated and the uninoculated plants. The uninoculated plants had no nodules; those grown in the presence of nitrate and inoculated had one or two nodules each; those grown in nitrate-free solution had about fifteen nodules each. Six weeks after planting, the plants grown in nitrate solution had thick, green leaves and thick roots, and no more nodules had developed on the inoculated plants. The plants grown in nitrate-free solution were yellowish except for the upper leaves, which were green; the roots were longer and more abundant than on the plants grown in nitrate solution. Nodules were abundant, continually developing on the new roots. The uninoculated plants in nitrate-free solution had no nodules and were practically dead.

The plants grown in nitrate-free solution with their roots exposed to light were slightly shorter than, and did not have quite as many nodules as, those grown in the same solution but with their roots kept in darkness. In the presence of the nitrate the development of certain green algæ interfered somewhat with root growth.

EXPERIMENT 4

INFLUENCE OF POTASSIUM NITRATE AND CALCIUM NITRATE IN PFEFFER'S SOLUTION

The procedure in this experiment was the same as in experiment 3. Only one plant was grown in each cylinder. Calcium nitrate was replaced by calcium chloride, and potassium nitrate was replaced by potassium chloride. The plants were kept until they began to blossom.

Results

In the solution with the nitrates, two or three nodules developed on each plant within twelve days after inoculation. No more nodules developed after that. In the cylinders not covered with black paper, algæ developed in abundance, and, surrounding the roots, dwarfed the plants. In the cylinders covered with black paper, also, some algæ developed in time, but they were far less abundant.

In the nitrate-free solution there was an abundant development of nodules. The nodules were more numerous on the plants grown in the cylinders covered with black paper than on those the roots of which were exposed to light. The root system of the plants grown in the nitrate-free solution was more developed than that of the plants grown in the nitrate solution.

An interesting point observed in this experiment and in experiment 3 was that the nodules developed, both in the nitrate solution and in the nitrate-free solution, immediately after inoculation. In the nitrate solution, however, no further development of nodules took place, while in the nitrate-free solution there was a continual development of new nodules on the new rootlets as time went on. This would seem to indicate either that the nodule-forming organisms were made inactive by the nature of the solution, or that the solution in some way affected the resisting power of the plants.

EXPERIMENT 5

INFLUENCE OF VARIOUS CONCENTRATIONS OF PFEFFER'S SOLUTION, BOTH WITH AND WITHOUT NITRATES

Wide-mouth bottles of 500 cubic centimeters capacity were used in this experiment. They were all covered with black paper. Two series were prepared, one with Pfeffer's full nutrient solution and the other with Pfeffer's solution in which the nitrates were replaced by the chlorides of the same metals. The following concentrations in each series were employed, taking the concentration of the full nutrient as 1: $\frac{1}{16}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3, 4, and 8. Five plants were grown in each bottle, and all were inoculated. The duration of the experiment was three weeks.

Results

In the full nutrient solution a few nodules developed in the $\frac{1}{16}$ concentration. In the other concentrations no nodules appeared within the three weeks.

In the nitrate-free solution nodules developed best in concentration 1. In the $\frac{1}{2}$ and $\frac{1}{4}$ concentrations a few nodules appeared.

EXPERIMENT 6

INFLUENCE OF PFEFFER'S SOLUTION IN WHICH THE ESSENTIAL ELEMENTS WERE ABSENT

In this experiment the same vessels were used as in experiment 5. The solutions were prepared as follows:

For solution minus nitrogen { $Ca(NO_3)_2$ was replaced by $CaCl_2$; KNO_3 was replaced by KCl

For solution minus potassium { KNO_3 was replaced by $NaNO_3$; KH_2PO_4 was replaced by NaH_2PO_4; KCl was replaced by NaCl

For solution minus phosphorus, KH_2PO_4 was replaced by NaH_2PO_4

For solution minus sulfur, $MgSO_4$ was replaced by $MgCl_2$

For solution minus magnesium, $MgSO_4$ was replaced by $MgCl_2$

For solution minus iron, $FeCl_3$ was replaced by NaCl

One plant was grown in each bottle. The plants were examined three weeks after planting.

Results

Nodules developed only in the nitrate-free solution. The plants in most of the solutions in this experiment and in experiment 5 did not grow well and normally. It is possible that slightly different results might have been obtained had the length of time of the experiment been extended. It was observed, however, in these and in the other experiments, that the number and the size of the nodules on a plant are influenced by the rate and the amount of growth of the plant. In other words, any disturbing factor in the normal functions of a plant tends to hinder the development of nodules.

EXPERIMENT 7

INFLUENCE OF MOISTURE

In experiments 7, 8, and 9, the plants were grown in glass tumblers. In each tumbler was placed 300 grams of air-dry sandy soil containing less than 0.5 per cent of moisture. The following percentages of moisture were used: 5, 10, 15, 20, 25, 30, 40, 50, and 60, the percentage being based on the air-dry soil. Three tumblers were used for each percentage of moisture, and five plants were grown in each tumbler. The plants were kept in the greenhouse. They were watered every other day, the necessary amount of water to be added being determined by weighing. The soil is naturally well inoculated with the Canada field pea organism, but in addition to this each tumbler was inoculated with a pure culture of the organism. The duration of the experiment was four weeks.

Results

The best growth took place in 15, 20, and 25 per cent of moisture. In 5 and 10 per cent of moisture the plants grew very slowly, while in 50 and 60 per cent the roots rotted. Nodules were present on all the plants. The number of the nodules on each plant increased with the percentage of moisture up to 40 per cent. These results agree with those of Gain (1893), who found that a larger number of nodules develop when the plants are abundantly watered.

EXPERIMENT 8

INFLUENCE OF CERTAIN SUBSTANCES IN VARYING QUANTITIES

The same soil and the same kind of vessels were used in this experiment as in experiment 7. Three hundred grams of the air-dry soil was in-

troduced into each glass tumbler; the substance to be tested was dissolved in the proper quantity of water, and this was added to each tumbler. Five plants were grown in each tumbler, and all the cultures were made in triplicate and were inoculated. The plants were allowed to grow for four weeks. The kind and the amount of the substance added, together with the results, are given in table 3:

TABLE 3. INFLUENCE ON NODULE DEVELOPMENT OF CERTAIN SUBSTANCES IN VARYING QUANTITIES

Substance used	Quantity added to 300 grams of soil (grams)	Condition of plants	Nodule development
KNO_3	0.25	Good growth	No nodules
	0.50	Poor growth	No nodules
	1.00	No growth	No nodules
	2.00	No growth	No nodules
$Ca(NO_3)_2$	0.25	Good growth	Few nodules
	0.50	Poor growth	No nodules
	1.00	No growth	No nodules
	2.00	No growth	No nodules
$MgSO_4$	0.25	Good growth	Nodules abundant
	0.50	Good growth	Nodules abundant
	1.00	Good growth	Nodules abundant
	2.00	Good growth	Nodules abundant
KH_2PO_4	0.25	Good growth	Nodules abundant
	0.50	Good growth	Nodules abundant
	1.00	Good growth	Nodules abundant
	2.00	Good growth	Nodules abundant
$CaCO_3$	0.25	Good growth	Nodules very abundant
	0.50	Good growth	Nodules very abundant
	1.00	Good growth	Nodules very abundant
	2.00	Good growth	Nodules very abundant
NH_4Cl	0.25	No growth	No nodules
	0.50	No growth	No nodules
	1.00	No growth	No nodules
	2.00	No growth	No nodules
$FeCl_3$	0.25	No growth	No nodules
	0.50	No growth	No nodules
	1.00	No growth	No nodules
	2.00	No growth	No nodules

TABLE 3 (*concluded*)

Substance used	Quantity added to 300 grams of soil (grams)	Condition of plants	Nodule development
Witte's peptone	0.25	Fair growth	Few nodules
	0.50	Poor growth	No nodules
	1.00	Poor growth	No nodules
	2.00	Poor growth	No nodules
Cane sugar	0.25	Good growth	Nodules present
	0.50	Good growth	Nodules present
	1.00	Good growth	Nodules present
	2.00	Good growth	Nodules present
	4.00	Poor growth	Nodules present
	8.00	Poor growth	No nodules
	16.00	Very poor growth	No nodules
Controls	Nothing added	Good growth	Nodules present

Results

The addition of $MgSO_4$, KH_2PO_4, and $CaCO_3$ in the concentrations used in the experiment had a beneficial effect on the development of nodules. Cane sugar at low concentrations had apparently no effect. At the concentrations of 4, 8, and 16 grams of sugar in 300 grams of soil, cane sugar was injurious, probably due to fermentation products and to stimulation of the development of microorganisms injurious to the plants and also to the development of nodules. The addition of NH_4Cl and $FeCl_3$ completely inhibited the growth of the plants. In the case of KNO_3 and $Ca(NO_3)_2$ the concentration of $\frac{1}{4}$ gram in 300 grams of soil had a beneficial effect on the growth of the plant, but an injurious effect on the development of nodules. A few nodules developed in the presence of $Ca(NO_3)_2$, but none in the presence of KNO_3. The higher concentrations of $Ca(NO_3)_2$ and KNO_3 inhibited nodule development and also caused injury to the plants.

EXPERIMENT 9

INFLUENCE OF CERTAIN ADDITIONAL SUBSTANCES

The method used in this experiment was the same as in experiment 8. Several additional chemicals were tested. In examining the plants, the

number of nodules was counted and both the total number of nodules and the average per plant for each concentration of the substance were calculated. The results follow in table 4:

TABLE 4. INFLUENCE OF CERTAIN ADDITIONAL SUBSTANCES ON NODULE DEVELOPMENT

Chemical used	Quantity added to 300 grams of soil (grams)	Number of plants	Condition of plants	Number of small nodules	Number of large nodules	Total number of nodules	Number of nodules per plant
$Ca(NO_3)_2$	0.25	10	Small	6	.....	6	0 6
	0.50	8	Small	.....	.....	0	0
	1.00	0	Plants killed	.....	.....	.....	.....
	2.00	0	Plants killed	.....	.....	.....	.....
Tannic acid	0.25	11	Good	40	138	178	16.2
	0.50	8	Good	50	41	91	11.4
	1.00	12	Good	17	50	67	5.6
	2.00	13	Good	100	20	120	9.2
KH_2PO_4	0.25	6	Good	10	50	60	10.0
	0.50	7	Good	50	58	108	15.4
	1.00	4	Small	30	10	40	10.0
	2.00	4	Small	16	5	21	5.3
$MgSO_4$	0.25	14	Good	46	100	146	10.4
	0.50	11	Good	26	50	76	6.9
	1.00	12	Good	60	41	101	8.4
	2.00	13	Good	24	32	56	4.3
KCl	0.25	7	Small	34	12	46	6.6
	0.50	0	Plants killed	.....	.....	.....	.....
	1.00	0	Plants killed	.....	.....	.....	.....
	2.00	0	Plants killed	.....	.....	.....	.....
KNO_3	0.25	14	Good	15	0	15	1.1
	0.50	14	Small	.....	.....	.....	.....
	1.00	3	Plants killed	.....	.....	.....	.....
	2.00	0	Plants killed	.....	.....	.....	.....
NH_4Cl	0.25	12	Good	0	0	0	0
	0.50	2	Small	0	0	0	0
	1.00	0	Plants killed	.....	.....	.....	.....
	2.00	0	Plants killed	.....	.....	.....	.....
Witte's peptone	0.25	11	Good	21	8	29	2.6
	0.50	11	Good	20	2	22	2.0
	1.00	9	Small	4	0	4	0.4
	2.00	1	Very small	.....	.....	.....	.....

TABLE 4 (*concluded*)

Chemical used	Quantity added to 300 grams of soil (grams)	Number of plants	Condition of plants	Number of small nodules	Number of large nodules	Total number of nodules	Number of nodules per plant
KOH	0.25	12	Good	35	50	85	7.1
	0.50	10	Good	10	45	55	5.5
	1.00	3	Small	11	25	36	12.0
	2.00	0	Plants killed	.....	.....	.....	.....
$Fe(NO_3)_3$	0.25	11	Good	4	0	4	0.4
	0.50	14	Good	1	0	1	0.1
	1.00	6	Small	0	0	0	0
	2.00	0	.....	.....	.....	.....	.....
$Ca(H_2PO_4)_2$	0.25	8	Good	6	45	51	6.4
	0.50	14	Good	90	94	184	13.1
	1.00	12	Good	54	60	114	9.5
	2.00	5	Small	8	11	19	3.8
$CaSO_4$	0.25	11	Good	22	65	87	7.9
	0.50	10	Good	15	60	75	7.5
	1.00	14	Good	57	50	107	7.4
	2.00	10	Good	21	45	66	6.6
$FeCl_3$	0.25	0	Plants killed	.....	.....	.....	.....
	0.50	0	Plants killed	.....	.....	.....	.....
	1.00	0	Plants killed	.....	.....	.....	.....
	2.00	0	Plants killed	.....	.....	.....	.....
Starch	1.00	12	Good	40	82	122	10.2
	2.00	13	Good	59	40	99	7.6
	4.00	8	Good	18	42	60	7.5
Controls	.....	18	Good	.....	.....	113	6.3
	.....	14	Good	.....	.....	107	7.6

Results

The results of experiment 9 are similar to those of experiment 8. The following chemicals added to the soil at the concentrations used in the experiment were injurious to the plants and tended to inhibit the development of nodules: $Ca(NO_3)_2$, KCl, KNO_3, NH_4Cl, Witte's peptone, $Fe(NO_3)_3$, and $FeCl_3$. On the other hand, tannic acid, KH_2PO_4, $MgSO_4$, KOH, $Ca(H_2PO_4)_2$, $CaSO_4$, and starch exerted a beneficial influence on nodule development and appeared to have no injurious effect on the plants.

GENERAL DISCUSSION OF RESULTS OF EXPERIMENTS

The nine experiments reported in Part II, on the factors affecting the development of nodules on Canada field peas, are not extensive enough to allow any broad and general deductions, but the results point to several conclusions. Nodules develop readily on Canada field peas in nutrient solutions, provided the proper nutrient salts are added. Varying the concentration appears to have a marked influence on nodule development. Aëration has no appreciable effect. Nodules developed on the long roots at as great a depth as 30 centimeters below the surface of the nutrient solution. If air is essential for the development of nodules, enough of it was dissolved in the nutrient solution under the conditions in the experiments. The presence of nitrates in the nutrient solution or in the soil tends to inhibit the development of nodules; the reason for this is not known. If the plants are grown in the presence of nitrates for about a week and then inoculated, a few nodules will develop, but no further development of nodules takes place; in water cultures without nitrates and inoculated, a continuous nodule development takes place as long as the roots grow.

The chemical composition of the various soils used for agricultural purposes differs. What influence this has on the various groups of the nodule-forming organism and on nodule development has never been extensively investigated. The results of the foregoing experiments emphasize its importance. The limited distribution of the different groups of the nodule-forming organism in some soils, the failures in inoculations, and the difficulty in growing certain legumes, may be explained in certain cases as being due to the composition of the soil. It is known that the addition of lime to certain soils has a beneficial effect on nodule development and on the growth of some legumes. It is highly probable that the addition of other substances to the soil may be beneficial to other legumes.

PART III. INFLUENCE OF VARIOUS MEDIA ON THE INFECTING POWER AND THE VITALITY OF BACILLUS RADICICOLA

As indicated previously, and again further developed in the discussion of this subject, the view has been maintained that the infecting power, or "virulence," of the nodule-forming organism may be impaired by cultivating it on certain media. In order to determine whether or not

such is the case, and also to determine the media most favorable for maintaining the vitality of the organism, the following experiments were made.

The term *virulence* has been used by previous investigators to mean the ability of the organism to penetrate the root and produce nodules. Since the term *virulence* in this connection, as also suggested by Edwards (in Marshall's Microbiologie), does not correctly apply to the legume bacteria, the term *infecting power* will be used throughout this paper.

Beyerinck (1890) was the first to isolate a pure culture of an organism from a nodule. Prazmowski (1890), Frank (1889), and Nobbe and others (1891), stimulated by Beyerinck's success, were also able to obtain pure cultures from the nodules of various legumes, and to produce nodules by inoculating the plants with the pure cultures. The experiments on the inoculation of legumes by pure cultures at once raised a question as to the classification of the nodule-forming organism, which question is intimately connected with the subject of the infecting power of the organism and the resistance of the plants. Do all the different organisms from the various species of legumes belong to several species, or do they belong only to one species but to several races or varieties? Can the organism from one species of legumes cause nodules on a different species of legumes? Is the relation between the legume and the organism a case of symbiosis or a case of parasitism? Does the organism have the biological or physiological character called *virulence* as understood by pathologists, and can this be altered or destroyed without injuring or destroying the other physiological activities? Do the host plants have a resistance in a pathological sense, and can this resistance be altered by the environmental factors without altering the morphology, the structure of the tissues, and the physiological activities of the plants? Is the resistance against the entrance of the organism into the root tissues different from the resistance against the development of the organism inside the root tissues? These and similar questions formed the foundation of the numerous investigations that were undertaken subsequently to the isolation of the pure culture of the nodule-forming organism, and nodule production by pure cultures.

Even before the isolation of the nodule-forming organism by Beyerinck, it was observed by Hellriegel (1886) that when peas, vetch, beans, clover, serradella, and lupines were inoculated with an infusion from the same

soil, all the plants developed nodules except the serradella and the lupine. From this Hellriegel inferred that important differences must exist between the nodule bacteria of the different legumes.

Beyerinck (1888) was of the opinion that the nodule-forming organisms of the legumes belonged to one species, but that there were several groups and in each group a number of varieties. From the results of his subsequent investigation (1890) he was forced to change his former opinion. He considered the organism of Ornithopus and that of Vicia to be two distinct species.

Frank's investigations (1899) led him to believe that there was only one species among the nodule-forming organisms.

Salfeld (1888) grew peas and horse beans in "Hochmoorboden," and inoculated one part of them with sandy soil in which peas were grown and the other part with sandy soil in which lupines were grown. Both the peas and the horse beans inoculated with the pea soil developed nodules, while those inoculated with the lupine soil were free from nodules.

Laurent (1901) could produce nodules on dwarf peas by inoculating them with material from nodules of thirty different leguminous species, but he claimed that the number, size, and appearance of the nodules was influenced by the inoculating material of the different sources.

Kirchner (1896) grew about one hundred different species of legumes in the Hohenheimer botanical garden. He observed that all the different species of legumes developed nodules in the garden soil except the soy beans, although these had been grown in the garden for ten years. The soy beans did not produce nodules until they were inoculated with soil on which Japanese soy beans had been grown.

The investigations of Mazé (1898) led him to divide the nodule-forming organisms into two groups — those adapted to a neutral or an alkaline soil, and those adapted to an acid soil; the former infecting the plants that favor neutral and alkaline soil, and the latter infecting the plants that favor acid soil.

Nobbe, Schmid, Hiltner, and Hotter (1891) undertook a very extensive series of investigations on the general subject of nitrogen assimilation by leguminous plants. Much of the present information on this subject is due to these men, especially to Hiltner and Nobbe. They showed that the only way to study the relations between the nodule-forming organisms of the different legumes and the different species and varieties of legumes

was to use pure cultures of the organisms for inoculation purposes, and not the soil infusions as was done by a number of previous investigators.

In a subsequent paper, Nobbe, Hiltner and Schmid (1895) arrived at the following important conclusions on the relation of nodule-forming organisms to the different species of legumes:

"The infecting power of the nodule bacteria of the various groups and species of legumes cannot be differentiated absolutely, but only in degree. The pure cultures from nodules of different species of legumes do not represent different species, but only different forms. We have not the least doubt that all the nodule bacteria of the different legumes we have studied, even those of Mimosæ, are one species, all belonging to *Bacillus radicicola* of Beyerinck. These bacteria, however, are influenced by the plants in whose roots they live to such a degree that their descendants are able to infect readily only that species of legumes to which the former host plant belonged, at the same time losing partly or completely the power to infect other species of legumes. When the legume is grown in a suitable soil, nodules will develop on the roots only when either those nodule bacteria are present which have lived previously on that legume species, or when the neutral nodule bacteria are present. The latter will be found in the soil where legumes have never been grown or where they have not been grown for a long time. If one legume is preponderantly grown in a soil, most of the neutral bacteria become influenced by this legume, and when a different legume is planted which is not closely related to the former no nodules will be formed, or only very few and faulty ones, and these will appear so late that they will be of very little value to the plants."[5]

By means of extensive experiments (Nobbe and Hiltner, 1896) it has been demonstrated that effective inoculation is obtained only when the plants are inoculated with bacteria from the nodules of the same species of legumes.

Moore (1905) conducted extensive cross-inoculation experiments, and maintains that "it is possible to cause the formation of nodules upon practically all legumes, no matter what was the source of the original organisms, provided they were cultivated for some time upon a synthetic nitrogen-free medium." He states further: "It is undoubtedly true that the long adaptation of the bacteria to the special conditions obtaining

[5] Translation from the original German.

in a particular species of legume enables such organisms to produce more abundant nodules in a shorter length of time than bacteria isolated from some other legume and grown upon nitrogen-free media. While this is of considerable practical importance, and will probably always make it necessary to distribute the specific organism for the specific crop, it does not in any way indicate that the bacteria found in the nodules of beans, peas, clovers, etc., are separate species. The most that can be maintained is that there is a slight physiological difference due to the long association with a plant of a peculiar reaction which enables the bacteria more easily to penetrate the host upon which they have been accustomed to grow. These slight racial characteristics can readily be broken down by cultivation in the laboratory, and it is entirely possible to secure a universal organism capable of producing a limited number of nodules upon all the legumes which now possess these growths."

Hopkins (1904) found that the organism from sweet clover readily inoculates alfalfa.

Nobbe and Hiltner (1900) undertook to train the nodule-forming organism of peas and that of beans so that the former may cause nodules on beans and the latter on peas. They succeeded in doing this, and drew the following conclusions:

1. The nodule-forming organism from peas can be trained to produce nodules on beans, and that from beans to produce nodules on peas.

2. Although some nodules are produced in both cases, the organisms do not assimilate any nitrogen at first.

3. If the pea organism that caused nodules on beans is isolated and beans are inoculated with it a second time, the organism then infects the beans more readily than at the first inoculation and its power to assimilate nitrogen is increased. The organism of beans behaves in the same manner when made to infect peas.

Kellerman (1912) reports that Mr. Leonard has succeeded in securing abundant inoculation on soy beans, lupines, and alfalfa from an organism of a culture originally isolated from the alfalfa nodule and kept on an artificial medium in the laboratory for about six years. Kellerman, therefore, is of the opinion that the nodule-forming organisms of all the Leguminosæ should be considered as a single species.

The evidence from the investigations mentioned above points to two conclusions: (1) that, with some exceptions, the nodule-forming organism

from one legume does not cause nodules on another legume; (2) that the organism from one legume may be trained to cause nodules on any other legume. The evidence for the latter conclusion, however, is not final.

About 1895 a German company placed nitragin on the market — a pure culture of the nodule-forming organism for inoculation purposes. The cultures were propagated on gelatin and their preparation was based on the results of the investigations of Nobbe and Hiltner. These cultures were extensively tested both in Germany and in other countries, and, as judged by the reports of these tests, the cultures proved only partially successful. As a result of these adverse reports on nitragin, Nobbe and Hiltner (1899 a) undertook to ascertain the cause of the low efficiency of their cultures. They had already shown that the nodule-forming organism can be trained to infect other legume species than that of its host plant, when they trained the organism from peas to produce nodules on beans and that from beans to cause nodules on peas. They went a step further and demonstrated that the infecting power of the organisms can be altered in degree. They stated that the propagation of the organism on artificial media increases or diminishes the vitality, and that in general nitrogenous media are injurious to the vitality of the organism.

Moore (1905) also reports that as a result of numerous trials it has been found that, although the bacteria increase most rapidly on a medium rich in nitrogen, the resulting growth is usually of very much reduced vitality, and when put into the soil these organisms have lost the ability to break up into the minute forms necessary in order to penetrate the root hairs.

In a further study of this subject, Hiltner (1900) was led to believe that this variableness in the infecting power of the nodule-forming organism is the determining factor of the number and size of the nodules on every plant when grown under otherwise favorable conditions. He took some older plants that already had nodules on their roots, and placed them in a nutrient solution without any nitrogen. Repeated inoculation with its own organism did not produce any nodules on the new rootlets. When fall came, and the leaves began to turn yellow and drop, and the organisms in the nodules became weaker than those in the solution, nodules began to develop on the rootlets. When Hiltner took older

plants that had no nodules on their roots and placed them in a similar solution to that used with the other plants, an immediate development of nodules took place on the new rootlets. From this and other experiments, Hiltner concluded that "the active nodules produce immunity in the plant against the bacteria that possess the same or a lower degree of infecting power than those already living in the nodules of that plant. Only bacteria of higher infecting power are able to enter the root tissue."[6]

Süchting (1904), believing that Hiltner's theory of the infecting power of the organism and its relation to nodule development was not sound, undertook a series of interesting experiments on this subject, as well as an elaborate discussion of Hiltner's theory and of his own theory. In his experiments Süchting attempted to ascertain three points: (1) Have the organisms that produce the first nodules on the plant less infecting power than those that produce nodules on the same plant subsequently? (2) Does the symbiosis with the plant influence the infecting power of the organism? (3) Does the artificial medium influence the infecting power of the organism?

From his experiments Süchting drew the following conclusions:

1. The infecting power of the bacteria is not proportional to the age of the nodule.

2. The passage of the bacteria through the host plant does increase their infecting power. Their infecting power does not vary at the different stages of the plant's vegetative period, and the feeding of the plant by potassium nitrate is injurious to the bacteria in the nodules.

3. When propagated on artificial media the lupine bacteria lose their infecting power on some media and may exist in a so-called pseudo form. On neutral media the bacteria retain their infecting power better than on acid media. The horse-bean bacteria do not behave in the same manner, but keep their infecting power for a long time on suitable media.

Lewis and Nicholson (1905), on the other hand, found by their experiments that "It seems that the presence or absence of nitrogen in the culture media is not the determining factor in maintaining the activity of the germ. Cultivation in the presence of the amount of nitrogen usually present in bouillon with from two to five per cent of cane sugar

[6] Translation from the original German.

or glucose, preferably the former, has given best results in a l of the work connected with the experiment."

In the following pages data are presented on experiments conducted through several years in an attempt to alter the "virulence"— that is, the infecting power — of the organism. In experiments 10, 12, and 13, *Bacillus radicicola* of Canada field pea was used; in experiment 11 that of alfalfa was used. The organisms were propagated and kept on various media. Their infecting power was tested and measured by the nodule development in plants grown in a sterilized sandy soil.

EXPERIMENT 10

INFLUENCE OF CLAY, LOAM, SAND, AND CARBORUNDUM

In this experiment the organism was grown on clay, sandy loam, sandy soil, fine quartz sand, coarse quartz sand, and carborundum. One hundred grams of each substance, air-dried, was introduced into Erlenmeyer flasks of 300 cubic centimeters capacity. After sterilization the media were heavily seeded with *B. radicicola*. This was accomplished by introducing into each flask the growth of *B. radicicola* from one agar slope, along with the necessary quantity of water. The amount of moisture added to each medium was about five per cent less than its capacity for holding water.

Two series of flasks were prepared. In series 1 the media, as soon as seeded with the organism, were spread on sterile paper and allowed to dry at room temperature. The time required for their complete drying was about six hours. In series 2 the media were left in the flasks, plugged with cotton, and allowed to stand in the laboratory. Drying of the media in this series was very gradual. The infecting power of the organism in these cultures was tested by inoculating plants. For this purpose Canada field peas were grown in sterilized soil in flowerpots, and were inoculated with the respective cultures at the time of seeding. Inoculation was accomplished by scattering one gram of the inoculating material over the soil in the flowerpots. The first test was made when the cultures were ten days old and the second test when the cultures were forty-six days old. When the plants were three weeks old they were examined for presence of nodules. The results are presented in tables 5 and 6:

TABLE 5. RESULTS OF FIRST INOCULATION TEST. THE CULTURES WERE TEN DAYS OLD

	Plants inoculated with	Number of plants	Number of plants with nodules	Total number of nodules	Number of nodules per plant
Series 1	Clay	17	4	31	1.8
	Sandy loam	23	13	81	3.5
	Sandy soil	14	7	45	3.2
	Fine quartz sand	15	3	9	0.6
	Coarse quartz sand	19	0	0	0
	Carborundum	11	2	25	2.3
Series 2	Clay	10	6	51	5.1
	Sandy loam	15	10	90	6.0
	Sandy soil	18	10	67	3.7
	Fine quartz sand	13	7	92	7.1
	Coarse quartz sand	18	12	94	5.2
	Carborundum	14	4	23	1.6
	Agar slope culture	19	15	90	4.7
	Controls	13	3	15	1.2

TABLE 6. RESULTS OF SECOND INOCULATION TEST. THE CULTURES WERE FORTY-SIX DAYS OLD

	Plants inoculated with	Number of plants	Number of plants with nodules	Total number of nodules	Number of nodules per plant
Series 1	Clay	5	5	53	10.6
	Sandy loam	12	11	79	6.6
	Sandy soil	12	10	151	12.6
	Fine quartz sand	10	10	58	5.8
	Coarse quartz sand	4	4	41	10.3
	Carborundum	11	7	113	10.3
Series 2	Clay	3	3	20	6.7
	Sandy loam	4	3	45	11.3
	Sandy soil	14	14	188	13.4
	Fine quartz sand	8	7	190	23.8
	Coarse quartz sand	7	6	78	11.1
	Carborundum	10	4	32	3.2
	Controls	36	13	120	3.3

Results

In both tests there was a certain amount of infection due to other sources than the inoculating materials. In the first test three of the thirteen plants used as controls developed nodules, while in the second test thirteen of the thirty-six control plants developed nodules. It was noticed, however, that, as a rule, if contamination took place subsequently to the inoculation and the plants were examined within four weeks after planting, the nodules due to the contamination were small and developed on the lateral roots near the surface of the soil, whereas nodules resulting from inoculation always appeared first on the taproot and were larger. Nevertheless, the results as shown in the two tables do not allow any clear-cut deductions. The plants of the first test (table 5) were examined three weeks after planting; the plants of the second test (table 6) were kept for four weeks, which probably accounts for the larger number of nodules on those plants.

It appears certain that *B. radicicola* remained alive and retained its infecting power in practically all the substances for forty-six days. Carborundum gave the poorest results. The plants inoculated with this substance developed only small nodules, mostly on the side roots — a fact pointing to subsequent infection. As regards the two series, much better inoculation was obtained from series 2 in both tests. In the first test 99 plants were inoculated with the cultures of series 1. These plants produced 191 nodules, which is an average of 1.9 nodules per plant. The 88 plants inoculated with the cultures from series 2 produced 417 nodules, which is an average of 4.7 nodules per plant. From similar calculations for the second test, it is found that the average number of nodules per plant was 9.2 in series 1, and 12 in series 2. In both tests the plants inoculated with the cultures of series 2 produced more nodules than those inoculated with the cultures of series 1. The drying of the substances in series 1 either reduced the infecting power of *B. radicicola*, or reduced the number of the organisms, or had both results.

EXPERIMENT 11

INFLUENCE OF HYDROCHLORIC ACID, SODIUM HYDROXIDE, AND CANE SUGAR, IN VARYING CONCENTRATIONS

In this experiment *B. radicicola* of alfalfa was isolated and identified according to the procedure described in Part I of this paper. The

organism was propagated on media 334, 335, and 337 (page 11), to which were added various amounts of hydrochloric acid (HCl) and sodium hydroxide (NaOH). Cane sugar was added in various amounts to medium 334.

Ten cubic centimeters of the media were introduced into each test tube and sterilized. While the agar was still melted the various additions were made to the tubes, and, after thorough mixing, the tubes were sloped.

All cultures were made in duplicate. At the end of three weeks stain preparations were made from each slope for morphological study, carbol fuchsin being used for staining. The media employed and the results of the morphological study are given in table 7:

TABLE 7. MORPHOLOGICAL VARIATION OF B. RADICICOLA ON THE DIFFERENT MEDIA

Medium	Morphological appearance	Multiplication
Medium 334, 10 cc.		
+ 0	Small, short cells	Fair
+ 0.1 cc. N/1 HCl	Short rods, slightly stained	Fair
+ 0.5 cc. N/1 HCl	Short rods, slightly stained	Poor
+ 1.0 cc. N/1 HCl	Short rods, slightly stained	None
+ 1.5 cc. N/1 HCl	Short rods, slightly stained	None
+ 2.0 cc. N/1 HCl	Short rods, slightly stained	None
+ 3.0 cc. N/1 HCl	Short rods, slightly stained	None
+ 0.1 cc. N/1 NaOH	Short rods, well stained	Good
+ 0.5 cc. N/1 NaOH	Short rods, well stained	Good
+ 1.0 cc. N/1 NaOH	Short rods, well stained	Poor
+ 1.5 cc. N/1 NaOH	Short rods, well stained	Poor
+ 2.0 cc. N/1 NaOH	Short rods, well stained, few small bacteroids	Very poor
+ 3.0 cc. N/1 NaOH	Short rods, well stained, few small bacteroids	Doubtful
+ 0.1 per cent cane sugar	Cells vary in size, bacteroids present	Good
+ 0.5 per cent cane sugar	Cells vary in size, bacteroids present	Good
+ 1.0 per cent cane sugar	Cells vary in size, bacteroids present	Good
+ 1.5 per cent cane sugar	Cells vary in size, bacteroids present	Good
+ 2.0 per cent cane sugar	Cells vary in size, bacteroids present	Good
+ 3.0 per cent cane sugar	Cells vary in size, bacteroids present	Good
+ 4.0 per cent cane sugar	Cells vary in size, bacteroids more abundant	Good
+ 5.0 per cent cane sugar	Cells vary in size, bacteroids more abundant	Good
+ 6.0 per cent cane sugar	Cells vary in size, bacteroids more abundant	Fair
+ 8.0 per cent cane sugar	Cells vary in size, bacteroids very abundant	Fair
+ 10.0 per cent cane sugar	Cells vary in size, bacteroids very abundant	Poor

TABLE 7 (*concluded*)

Medium	Morphological appearance	Multiplication
Medium 335, 10 cc.		
+ 0	Variation in size and shape, few bacteroids	Good
+ 0.1 cc. N/1 HCl	Variation in size and shape, few bacteroids	Good
+ 0.5 cc. N/1 HCl	Very few rods	Poor
+ 1.0 cc. N/1 HCl	No organisms visible	Doubtful
+ 1.5 cc. N/1 HCl	No organisms visible	None
+ 2.0 cc. N/1 HCl	No organisms visible	None
+ 3.0 cc. N/1 HCl	No organisms visible	None
+ 0.1 cc. N/1 NaOH	Variation in size and shape, few bacteroids	Very good
+ 0.5 cc. N/1 NaOH	Variation in size and shape, few bacteroids	Very good
+ 1.0 cc. N/1 NaOH	Greater variation, more bacteroids than in 0.5	Good
+ 1.5 cc. N/1 NaOH	Greater variation, more bacteroids than in 0.5	Good
+ 2.0 cc. N/1 NaOH	Greater variation, more bacteroids than in 0.5	Poor
+ 3.0 cc. N/1 NaOH	Cells stained deeper than others, bacteroids present	Doubtful
Medium 337, 10 cc.		
+ 0	Pronounced variation in shape and size of cells.	Good
+ 0.1 cc. N/1 HCl	Small cells, irregular shape and size	Good
+ 0.5 cc. N/1 HCl	Very slender cells, irregular shape and size	Poor
+ 1.0 cc. N/1 HCl	Extremely small cells	Very poor
+ 1.5 cc. N/1 HCl	Extremely small cells	Doubtful
+ 2.0 cc. N/1 HCl	Extremely small cells	None
+ 3.0 cc. N/1 HCl	No organisms visible	None
+ 0.1 cc. N/1 NaOH	Short, irregular cells	Abundant
+ 0.5 cc. N/1 NaOH	Short, irregular cells	Abundant
+ 1.0 cc. N/1 NaOH	Shape and size extremely varied, many bacteroids	Abundant
+ 1.5 cc. N/1 NaOH	Large cells, varying in shape and size, many bacteroids	Good
+ 2.0 cc. N/1 NaOH	Large cells, varying in shape and size, many bacteroids	Good
+ 3.0 cc. N/1 NaOH	Very large cells, varying in shape and size, many bacteroids	Poor

In order to test the infecting power of the different cultures, three flowerpots of alfalfa plants grown in sterile soil were inoculated with the organism from each test tube. Four weeks after inoculation the plants were examined for nodule development. The results of the inoculations are presented in table 8. Fifty-eight plants in flowerpots were used as controls. These plants were grown in sterile soil and were not inoculated. The control plants were distributed among the other

plants in order to see to what extent an infection from neighboring flower-pots may take place.

TABLE 8. INFECTING POWER OF VARIOUS CULTURES. B. RADICICOLA WAS PROPAGATED FOR THREE WEEKS ON THE DIFFERENT MEDIA

Medium	Number of plants inoculated	Total number of nodules	Number of nodules per plant
Medium 334, 10 cc.			
+ 0	42	265	6.3
+ 0.1 cc. N/1 HCl	50	320	6.4
+ 0.5 cc. N/1 HCl	43	188	4.3
+ 1.0 cc. N/1 HCl	32	0	0
+ 1.5 cc. N/1 HCl	38	6	0.2
+ 2.0 cc. N/1 HCl	53	22	0.4
+ 3.0 cc. N/1 HCl	35	7	0.2
+ 0	37	184	5.0
+ 0.1 cc. N/1 NaOH	43	275	6.4
+ 0.5 cc. N/1 NaOH	48	234	4.9
+ 1.0 cc. N/1 NaOH	47	304	6.5
+ 1.5 cc. N/1 NaOH	34	168	4.9
+ 2.0 cc. N/1 NaOH	27	94	3.5
+ 3.0 cc. N/1 NaOH	27	75	2.8
+ 0.1 per cent cane sugar	46	182	4.0
+ 0.5 per cent cane sugar	35	124	3.5
+ 1.0 per cent cane sugar	32	117	3.7
+ 1.5 per cent cane sugar	48	198	4.1
+ 2.0 per cent cane sugar	38	140	3.7
+ 3.0 per cent cane sugar	29	125	4.3
+ 4.0 per cent cane sugar	35	138	3.9
+ 6.0 per cent cane sugar	33	132	4.0
+ 8.0 per cent cane sugar	33	110	3.3
+ 10.0 per cent cane sugar	30	85	2.8
Medium 335, 10 cc.			
+ 0	29	132	4.6
+ 0.1 cc. N/1 HCl	34	183	5.4
+ 0.5 cc. N/1 HCl	50	6	0.1
+ 1.0 cc. N/1 HCl	68	0	0
+ 1.5 cc. N/1 HCl	50	22	0.4
+ 2.0 cc. N/1 HCl	56	5	0.1
+ 3.0 cc. N/1 HCl	58	4	0.1
+ 0.1 cc. N/1 NaOH	33	186	5.6
+ 0.5 cc. N/1 NaOH	32	131	4.1
+ 1.0 cc. N/1 NaOH	30	212	7.1
+ 1.5 cc. N/1 NaOH	36	284	7.9
+ 2.0 cc. N/1 NaOH	30	180	6.0
+ 3.0 cc. N/1 NaOH	21	109	5.2

TABLE 8 (*concluded*)

Medium	Number of plants inoculated	Total number of nodules	Number of nodules per plant
Medium 337, 10 cc.			
+ 0	23	54	2.3
+ 0.1 cc. N/1 HCl	32	50	1.6
+ 0.5 cc. N/1 HCl	37	110	3.0
+ 1.0 cc. N/1 HCl	36	77	2.1
+ 1.5 cc. N/1 HCl	36	23	0.6
+ 2.0 cc. N/1 HCl	33	94	2.8
+ 3.0 cc. N/1 HCl	32	132	4.1
+ 0.1 cc. N/1 NaOH	26	80	3.1
+ 0.5 cc. N/1 NaOH	27	120	4.4
+ 1.0 cc. N/1 NaOH	39	174	4.5
+ 1.5 cc. N/1 NaOH	31	114	3.7
+ 2.0 cc. N/1 NaOH	32	154	4.8
+ 3.0 cc. N/1 NaOH	33	87	2.6
Controls	735	*624	0.8

* Nodules were present in only thirteen out of fifty-eight pots

Results

The extent of multiplication and the description of the morphological characters of the organisms in the various media are summarized in table 7. No visible increase in the number of organisms was found when 1 cubic centimeter or more of the normal solution of HCl was added to 10 cubic centimeters of each medium. The few organisms found on these slopes were probably those that were introduced when the slopes were inoculated. In medium 337 slight multiplication took place on slopes of 10 cubic centimeters of the medium plus 1 cubic centimeter of the normal solution of HCl. The best multiplication occurred in media 335 and 337 when 0.1 to 1 cubic centimeter of the normal solution of NaOH was added to 10 cubic centimeters of the medium. This was particularly noticeable in medium 337. The addition of 3 cubic centimeters of the normal solution of NaOH to 10 cubic centimeters of the medium practically inhibited multiplication. The bacteroids developed more readily when NaOH was added.

The addition of sugar to medium 334, up to 10 per cent, caused more rapid multiplication and also the development of bacteroids.

In medium 334 multiplication of the organisms was very slow and only a few bacteroids developed in the three weeks. In medium 337 multiplication was abundant and bacteroids developed.

The results of inoculation are given in table 8. There were two hundred and eight flowerpots, which were crowded together because of lack of space. Fifty-eight flowerpots contained control plants, which were scattered among the inoculated pots. The plants in forty-five of the control pots developed no nodules; thirteen of the control pots were contaminated, and these were located mostly among the flowerpots inoculated with the organisms grown on medium 337. This contamination occurred in spite of the precautions taken to prevent the organisms from being carried from one flowerpot to another when the plants were watered. In these experiments and in others not reported in this paper, it was found that when a large number of flowerpots were used at one time it was difficult to prevent infection from other sources than that of the inoculating material. This was particularly true in the case of those legumes that produce an abundance of nodules in the soil of this region, and when the plants were allowed to grow for longer periods than three weeks. The results in this experiment are marred by a certain amount of contamination. The data in table 8 are so arranged as to show the total number of nodules on all the plants inoculated with the same material, and also to show the average number of nodules to each of these plants. Basing the conclusions on the mere number of nodules overemphasizes the importance of the contamination. When the plants were examined, and the size, location, and evenness of distribution of the nodules among the plants in the same flowerpot were noted, in addition to their total number, much more reliable evidence was obtained. In view of this, the following conclusions seemed to be warranted:

When *B. radicicola* of alfalfa is propagated on media 334, 335, and 337 and kept for three weeks, multiplication of the organism takes place and the infecting power is not lost. A slight reduction in infecting power seems to be apparent on medium 337. The addition of 0.1 cubic centimeter of the normal solution of HCl to 10 cubic centimeters of each of these media very slightly diminished the amount of growth, but the infecting power was not affected. When 0.5 cubic centimeter of the acid solution

was added, the amount of growth was decidedly reduced on media 334 and 335 and the infecting power also was reduced. The influence of this amount of acid in medium 337 was not so pronounced. The addition of 1 cubic centimeter or more of the acid solution completely inhibited the growth and the infecting power of the organism on all three media.

The addition of 0.1, 0.5, 1, 1.5, and 2 cubic centimeters, respectively, of the normal solution of NaOH, to 10 cubic centimeters of each of the media, increased the amount of growth and also the effectiveness of the inoculation. The addition of 3 cubic centimeters of the normal solution of NaOH reduced the amount of growth and the infecting power. The heaviest growth took place on medium 337 to which the above amounts of NaOH were added, but the plants inoculated with these cultures did not produce as many nodules as did the plants inoculated with the organisms propagated on media 334 and 335 — a fact that suggests the possibility of a reduction in infecting power.

The addition of cane sugar to medium 334 in the amounts indicated in table 8 has a beneficial influence on the multiplication of the organism. The infecting power does not seem to be affected by it. All the cultures in which any multiplication was observed produced positive inoculation, so that the infecting power of the organism was not destroyed. The variations in the number of nodules on the plants inoculated by the different cultures seem to indicate that the organisms propagated on medium 337 partly lost their infecting power. This measure of infecting power, however, is not accurate, and other explanations might easily be supplied. The most noticeable point was that positive inoculation was produced by all the cultures in which any multiplication took place, so that the growth and the infecting power seem to run parallel.

EXPERIMENT 12

INFLUENCE OF SOME OTHER MEDIA

The organism, isolated as described in Part I, was kept in the laboratory for two years on agar slopes, medium 335, before this experiment was started. During this time the organism was continually exposed to diffused light and to the ordinary variations in temperature and humidity. The organism was then propagated on agar slopes, medium 334, to which various substances were added as is indicated in tables 9 and 10. Twelve

TABLE 9. DESCRIPTION OF THREE-WEEKS-OLD GROWTH OF B. RADICICOLA ON AGAR SLOPES

Medium 334 with	Description of growth
2.0 per cent cane sugar	Good, watery
2.0 per cent dextrose	Good, watery
2.0 per cent levulose	Not visible
2.0 per cent lactose	Not so good as with cane sugar
2.0 per cent galactose	Good, watery
10.0 per cent cane sugar	Good, watery
20.0 per cent cane sugar	Good, watery
40.0 per cent cane sugar	Slight, very transparent
2.0 per cent glycerin	Good, watery
2.0 per cent mannite	Good, watery
0.1 per cent asparagin	Fair
1.0 per cent salicin	Fair, whitish, not watery
0.5 per cent amygdalin	Fair, whitish
0.5 per cent resorcin	Hardly visible
0.2 per cent phloroglucin	Not visible
0.5 per cent potassium oxalate	Not visible
0.5 per cent potassium citrate	Not visible
0.1 per cent potassium nitrate	Poor, fluorescent, opaque
0.2 per cent potassium nitrate	Poor, fluorescent, opaque
0.6 per cent potassium nitrate	Poor, fluorescent, opaque
0.1 per cent calcium nitrate	Medium, very opaque, fluorescent, tough, brownish
0.2 per cent calcium nitrate	Medium, very opaque, fluorescent, tough, brownish
0.6 per cent ca cium nitrate	Medium, very opaque, fluorescent, tough, brownish
1.0 per cent gelatin	Medium, juicy, opaque
5.0 per cent gelatin	Poor
1.0 per cent Witte's peptone	Abundant, juicy
2.0 per cent Witte's peptone	Abundant, juicy
5 0 per cent Witte's peptone	Not visible
0.2 per cent Merck's peptone	Abundant, fluorescent, whitish to brownish
1.0 per cent Merck's peptone	Abundant, fluorescent, whitish to brownish
3 0 per cent Merck's peptone	Not visible

tubes of each medium were prepared, and after being sterilized they were sloped and inoculated.

The following substances were used alone as media: soy bean hay, ground; soy bean roots, ground; Canada field pea hay, ground; Canada field pea roots, ground; Canada field pea seeds, ground; compost (well-decomposed cow feces), ground; partly decomposed cow feces, ground; fresh cow feces, ground; corn meal; sawdust; wheat bran; wheat middlings; sandy soil; muck; cornstarch. These substances were dried at 100° C.,

ground fine, and then introduced into test tubes 25 x 180 millimeters in size. Each tube was filled to one-third its capacity. Twelve test tubes were prepared for each medium, plugged with cotton, and sterilized in the autoclave for one hour at 15° C. For inoculation 10 cubic centimeters of sterile water in which the organisms were suspended was added to each test tube. At the end of two weeks the test tubes were sealed with paraffin in order to reduce evaporation. All the cultures were kept in the laboratory at room temperature.

Three tests were made in order to determine the infecting power of the organism propagated on the various media. The first test was made at the end of the third week, the second at the end of the tenth week, and the third at the end of the twentieth week. In these tests one test tube was taken from each of the media, the total number of organisms in each of these tubes was determined by the plate method, and Canada field pea plants were inoculated.

The plants were grown in sterilized sandy soil in flowerpots, three flowerpots being inoculated with each culture. When the plants were three weeks old they were examined and the nodules on each plant were counted. In table 9 is given a description of the three-weeks-old growth

TABLE 10. NUMBER OF ORGANISMS IN THE VARIOUS MEDIA AT THE TIME THE TESTS WERE MADE

Medium	Number of organisms per gram		
	Cultures three weeks old	Cultures ten weeks old	Cultures twenty weeks old
Soy bean hay	0	0	0
Soy bean roots	0	0	0
Canada field pea hay	Few	0	0
Canada field pea roots	1,500,000,000	306,000,000	0
Canada field pea seeds	10,000,000,000	185,000,000	.....
Compost	340,000,000	75,000,000	120,000,000
Partly decomposed cow feces	120,000,000	75,000,000	3,240,000,000
Fresh cow feces	60,000,000	440,000,000	810,000,000
Corn meal	7,200,000,000	Contamination	97,000,000
Sawdust	32,000,000	3,000,000	3,500,000
Wheat bran	10,000,000,000	370,000,000	600,000,000
Wheat middlings	4,000,000,000	390,000,000	420,000,000
Sandy soil	60,000,000	37,000,000	20,000,000
Muck	620,000,000	222,000,000	240,000,000
Cornstarch	200,000,000	55,000,000	97,000,000

TABLE 10 (*concluded*)

Medium 334 with	Number of organisms on one agar slope		
	Cultures three weeks old	Cultures ten weeks old	Cultures twenty weeks old
2.0 per cent cane sugar	No count made	300,000,000	90,000,000
10.0 per cent cane sugar	No count made	3,000,000	5,000,000
20.0 per cent cane sugar	No count made	1,000,000	10,000
40.0 per cent cane sugar	No count made	16,000,000	No colonies
2.0 per cent dextrose	No count made	31,000,000	52,000,000
2.0 per cent levulose	No count made	400,000	No colonies
2.0 per cent lactose	No count made	Plates spoiled	180,000
2.0 per cent galactose	No count made	3,000,000	600,000
2.0 per cent glycerin	No count made	250,000,000	20,000,000
2.0 per cent mannite	No count made	.....	25,000,000
0.1 per cent asparagin	No count made	300,000,000	108,000,000
1.0 per cent salicin	No count made	No colonies	No colonies
0.5 per cent amygdalin	No count made	250,000,000	No colonies
0.5 per cent resorcin	No count made	No colonies	No colonies
0.2 per cent phloroglucin	No count made	Few	No colonies
0.1 per cent potassium nitrate	No count made	120,000,000	72,000,000
0.2 per cent potassium nitrate	No count made	74,000,000	224,000,000
0.6 per cent potassium nitrate	No count made	259,000,000	144,000,000
0.1 per cent calcium nitrate	No count made	280,000,000	486,000,000
0.2 per cent calcium nitrate	No count made	150,000,000	108,000,000
0.6 per cent calcium nitrate	No count made	400,000,000	130,000,000
1.0 per cent gelatin	No count made	150,000,000	64,000,000
5.0 per cent gelatin	No count made	50,000,000	Plates spoiled
1.0 per cent Witte's peptone	No count made	50,000	216,000,000
2.0 per cent Witte's peptone	No count made	Contaminated	648,000,000
5.0 per cent Witte's peptone	No count made	No colonies	No colonies
0.2 per cent Merck's peptone	No count made	22,000,000	8,000,000
1.0 per cent Merck's peptone	No count made	300,000,000	324,000,000
3.0 per cent Merck's peptone	No count made	No colonies	No colonies
0.5 per cent potassium oxalate	No count made	No colonies	No colonies
0.5 per cent potassium citrate	No count made	No colonies	No colonies

of *B. radicicola* on the agar slopes to which the various chemicals were added. The number of organisms in the cultures on the various media at the time they were tested for their efficiency to produce nodules is given in table 10. The results of the inoculations are shown in tables 11, 12, and 13.

Results

Growth and number of organisms (tables 9 and 10)

Bacillus radicicola of Canada field pea produces scant growth on agar

slope medium 334. Some of the substances that were added to this medium retarded or completely inhibited the growth; others had no appreciable effect on the growth; and still others caused a decided increase in the growth as compared with that on medium 334 alone. (Table 9.) No visible growth was produced when the following substances were added: levulose 2 per cent, phloroglucin 0.2 per cent, potassium oxalate 0.5 per cent, potassium citrate 0.5 per cent, Witte's peptone 5 per cent, Merck's peptone 3 per cent. The growth in the remaining cultures was watery, almost transparent, very viscous, especially in the presence of sugars.

The number of organisms on the agar slopes was determined when the cultures were ten and twenty weeks old, and in the case of the substances in which the organism was propagated the determination was made at three, ten, and twenty weeks. The data in table 10 show a wide variation; but in general, wherever a visible amount of growth appeared on the agar slopes large numbers of organisms were found. At the end of ten weeks no organisms were found in the presence of salicin 1 per cent, resorcin 0.5 per cent, Witte's peptone 5 per cent, Merck's peptone 3 per cent, potassium oxalate 0.5 per cent, and potassium citrate 0.5 per cent. At the end of twenty weeks, in addition to the above no organisms were found in the presence of cane sugar 40 per cent, levulose 2 per cent, amygdalin 0.5 per cent, and phloroglucin 0.2 per cent.

Very large numbers of the organism were found on most of the substances that were ground and used as media. Wheat bran and ground Canada field pea seeds each had 10,000,000,000 organisms per gram at the end of three weeks. The organisms remained in a vigorous condition on these media for twenty weeks, as judged by the development of colonies and by the results of inoculation of the plants. In soy bean hay and soy bean roots no multiplication took place, and the organisms introduced at the time of seeding these two media were found to be dead at the end of three weeks.

First test of infecting power (table 11)

Three flowerpots were inoculated with each culture, these cultures being three weeks old when used. The inoculated plants were kept in the greenhouse and were examined for nodule development three weeks after inoculation. The data are presented in table 11:

TABLE 11. INFECTING POWER OF CULTURES THREE WEEKS OLD

Medium 334 with	Nodules in flower-pot 1	Nodules in flower-pot 2	Nodules in flower-pot 3
2.0 per cent cane sugar	Many	Many	Many
2.0 per cent dextrose	Many	Many	Few
2.0 per cent levulose	None	None	None
2.0 per cent lactose	Many	Many	Many
2.0 per cent galactose	Many	Many	Many
10.0 per cent cane sugar	Many	Many	Few
20.0 per cent cane sugar	Few	Few	Few
30.0 per cent cane sugar	Few	Few	Many
40.0 per cent cane sugar	Few	Few	Few
1.0 per cent salicin	Many	Many	None
0.5 per cent amygdalin	Many	Few	Few
0.5 per cent resorcin	None	None	Few
0.2 per cent phloroglucin	Few	Few	Few
0.5 per cent potassium oxalate	None	None	None
0.5 per cent potassium citrate	None	None	None
0.1 per cent potassium nitrate	Many	Many	Many
0.2 per cent potassium nitrate	Many	Many	Few
0.6 per cent potassium nitrate	Few	Few	Few
0.1 per cent calcium nitrate	Many	Many	Few
0.2 per cent calcium nitrate	Many	Many	Few
0.6 per cent calcium nitrate	Many	Many	Many
1.0 per cent Witte's peptone	Many	Many	Many
2.0 per cent Witte's peptone	Few	Few	None
5.0 per cent Witte's peptone	None	None	None
0.2 per cent Merck's peptone	Many	Many	Many
1.0 per cent Merck's peptone	Many	Many	Many
3.0 per cent Merck's peptone	None	None	None
Other media			
Soy bean hay	None	None	None
Soy bean roots	Few	Few	Few
Canada field pea hay	Few	Few	Few
Canada field pea roots	Many	Many	Many
Sandy soil	Many	Many	Many
Muck	Many	Many	Many
Sawdust	Many	Many	Many
Wheat bran	Many	Many	Many
Wheat middlings	Many	Many	Many
Corn meal	Many	Many	Many
Ground field peas	Many	Many	Many
Compost	Many	Many	Many
Partly decomposed cow feces	Many	Many	Many
Fresh cow feces	Many	Many	Many
Controls	None	None	None
Controls	None	None	None
Controls	None	None	None
Controls	None	None	None
Controls	None	None	None

As far as could be ascertained by these results, there was no reduction in the infecting power of the organisms when they were propagated and kept on the above media for three weeks. When these results are compared with the data in tables 9 and 10, it is seen that those cultures in which no visible growth took place produced either no inoculation or very poor inoculation, and that the cultures in which the organisms multiplied produced good inoculation.

Second test of infecting power (table 12)

In this test the cultures were ten weeks old. The exact number of nodules on each plant was counted, and from these figures calculations were made of (1) the total number of nodules in each flowerpot, (2) the average number of nodules per plant in each flowerpot, and (3) the average number of nodules per plant of all the plants inoculated with the same culture. It was hoped that the averages would give a more nearly accurate measure of the infecting power of the different cultures. The data are given in table 12. Fifteen plants were inoculated with each culture, five plants being grown in each flowerpot.

TABLE 12. INFECTING POWER OF CULTURES TEN WEEKS OLD

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Laboratory number of flowerpots	Number of nodules on each plant					Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5			
1	Medium 334 + 2 per cent cane sugar	1	14	4	1	0	12	31	6.2	
		2	22	10	2	9	12	55	11.0	8.6
		3	....	....	....	....	....	.....	.....	
2	Medium 334 + 2 per cent cane sugar	4	6	0	3	10	4	23	4.6	
		5	12	4	15	12	10	53	10.6	7.5
		6	14	7	6	2	7	36	7.2	
4	Medium 334 + 10 per cent cane sugar	7	5	7	14	12	12	50	10.0	
		8	10	8	14	8	....	40	10.0	10.2
		9	10	15	18	5	5	53	10.6	

TABLE 12 (*continued*)

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Laboratory number of flowerpots	Number of nodules on each plant					Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5			
6	Medium 334 + 20 per cent cane sugar	10	8	1	8	2	3	22	4.4	3.7
		11	15	5	1	0	5	26	5.2	
		12	4	0	4	0	0	8	1.6	
7	Medium 334 + 40 per cent cane sugar	13	11	8	9	5	....	33	8.2	9.2
		14	18	12	8	16	....	54	13.5	
		15	8	3	8	8	6	33	6.6	
8	Medium 334 + 2 per cent dextrose	16	0	14	15	10	9	48	9.6	8.7
		17	10	12	14	7	....	43	10.7	
		18	4	8	4	6	....	22	5.5	
9	Medium 334 + 2 per cent lactose	19	0	3	1	5	....	9	2.2	2.1
		20	3	1	0	2	3	9	1.8	
		21	0	6	5	0	0	11	2.2	
10	Medium 334 + 2 per cent galactose	22	5	8	7	....	....	20	6.7	9.7
		23	10	11	16	4	....	41	10.2	
		24	8	9	10	15	14	56	11.2	
12	Medium 334 + 2 per cent glycerin	28	3	2	16	14	14	49	9.8	12.1
		29	15	15	20	10	....	60	15.0	
		30	....	....	....	....	....	.....	.....	
13	Medium 334 + 0.5 per cent potassium oxalate	31	0	0	0	0	0	0	0	0.6
		32	0	2	3	4	....	9	2.2	
		33	0	0	0	0	0	0	0	
14	Medium 334 + 0.2 per cent phloroglucin	34	0	0	0	3	....	3	0.7	2.2
		35	0	0	0	0	....	0	0	
		36	1	3	2	5	15	26	5.2	
15	Medium 334 + 0.5 per cent resorcin	37	5	5	5	8	....	23	5.7	2.6
		38	1	4	0	6	....	11	2.7	
		39	0	0	0	0	0	0	0	
16	Medium 334 + 1 per cent salicin	40	0	2	4	0	3	9	1.8	3.0
		41	10	0	0	5	12	27	5.4	
		42	0	2	0	4	....	6	1.5	
17	Medium 334 + 0.5 per cent amygdalin	43	0	8	14	2	16	40	8.0	12.0
		44	10	15	24	15	8	72	14.4	
		45	14	20	10	....	....	44	14.7	

TABLE 12 (*continued*)

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Laboratory number of flowerpots	Number of nodules on each plant					Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5			
18	Medium 334 + 0.1 per cent asparagin	46	16	10	6	18	10	60	12.0	8.8
		47	10	5	8	10	....	33	8.2	
		48	6	0	12	2	10	30	6.0	
19	Medium 334 + 0.1 per cent potassium nitrate	49	7	3	10	12	15	47	9.4	6.9
		50	12	5	6	0	0	23	4.6	
		51	13	15	0	0	5	33	6.6	
20	Medium 334 + 0.2 per cent potassium nitrate	52	2	2	6	9	10	29	5.8	5.3
		53	1	8	8	12	1	30	6.0	
		54	0	2	1	8	10	21	4.2	
21	Medium 334 + 0.6 per cent potassium nitrate	55	10	16	10	18	2	56	11.2	10.6
		56	13	16	2	30	....	61	15.2	
		57	12	0	5	2	12	31	6.2	
22	Medium 334 + 0.1 per cent calcium nitrate	58	3	2	5	10	8	28	5.6	6.7
		59	6	6	3	10	8	33	6.6	
		60	8	0	14	5	12	39	7.8	
23	Medium 334 + 0.2 per cent calcium nitrate	61	12	12	10	5	14	53	10.6	13.1
		62	6	6	20	18	22	72	14.4	
		63	16	12	20	8	16	72	14.4	
24	Medium 334 + 0.6 per cent calcium nitrate	64	12	4	3	0	10	29	5.8	5.2
		65	3	2	7	7	9	28	5.6	
		66	0	0	6	10	5	21	4.2	
25	Medium 334 + 0.5 per cent potassium citrate	67	4	2	3	1	....	10	2.5	0.7
		68	0	0	0	0	0	0	0	
		69	0	0	0	0	0	0	0	
26	Medium 334 + 0.2 per cent Merck's peptone	70	4	7	8	4	4	27	5.4	4.4
		71	0	2	5	5	....	12	3.0	
		72	0	0	5	7	10	22	4.4	
27	Medium 334 + 1 per cent Merck's peptone	73	0	2	8	7	14	31	6.2	6.3
		74	20	7	0	0	8	35	7.0	
		75	7	8	0	12	2	29	5.8	
28	Medium 334 + 1 per cent Witte's peptone	76	14	0	10	0	15	39	7.8	15.8
		77	14	35	20	30	....	99	24.7	
		78	5	30	25	7	....	67	16.7	

TABLE 12 (*continued*)

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Laboratory number of flowerpots	Number of nodules on each plant					Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5			
29	Medium 334 + 2 per cent Witte's peptone	79	0	2	0	4	0	6	1.2	
		80	2	3	4	0	1	10	2.0	1.6
		81	0	1	5	0	....	6	1.5	
30	Medium 334 + 5 per cent Witte's peptone	82	0	0	0	0	0	0	0	
		83	2	0	0	4	0	6	1.2	0.4
		84	0	0	0	0	0	0	0	
31	Medium 334 + 5 per cent gelatin	85	10	14	16	35	34	109	21.8	
		86	2	8	10	16	6	42	8.4	13.9
		87	0	18	5	10	25	58	11.6	
32	Soy bean roots, ground	88	0	0	0	3	0	3	0.6	
		89	0	0	1	7	....	8	2.0	0.9
		90	2	0	0	0	0	2	0.4	
33	Soy bean hay, ground	91	0	0	0	0	0	0	0	
		92	0	0	0	0	0	0	0	0
		93	0	0	0	0	0	0	0	
34	Canada field pea roots, ground	94	0	0	5	8	6	19	3.8	
		95	8	10	6	12	10	46	9.2	7.0
		96	3	20	5	8	4	40	8.0	
35	Canada field pea hay, ground	97	12	5	2	8	....	27	6.7	
		98	0	0	7	2	1	10	2.0	2.6
		99	0	0	0	0	0	0	0	
36	Sawdust	100	1	0	0	0	0	1	0.2	
		101	10	0	7	6	0	23	4.6	1.6
		102	0	0	0	0	0	0	0	
37	Corn meal	103	0	0	0	0	0	0	0	
		104	0	0	0	0	0	0	0	0.1
		105	0	1	0	0	0	1	0.2	
38	Cornstarch	106	2	6	0	12	16	36	7.2	
		107	18	6	2	12	....	38	9.5	9.9
		108	7	15	8	15	20	65	13.0	
39	Wheat bran	109	18	0	8	8	6	40	8.0	
		110	8	8	8	14	8	46	9.2	8.6
		111	....	....	....	....	....	.....	.....	

TABLE 12 (*concluded*)

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Laboratory number of flowerpots	Number of nodules on each plant					Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5			
40	Wheat middlings	112	6	18	20	4	10	58	11.6	
		113	20	12	5	6	10	53	10.6	9.8
		114	12	0	7	3	14	36	7.2	
41	Compost	115	16	3	0	0	....	19	4.7	
		116	12	4	15	8	0	39	7.8	8.4
		117	10	14	12	16	8	60	12.0	
42	Partly decomposed cow feces	118	5	6	7	5	10	33	6.6	
		119	6	3	14	8	12	43	8.6	7.4
		120	8	6	3	4	14	35	7.0	
43	Fresh cow feces	121	0	0	7	0	....	7	1.7	
		122	3	7	20	5	12	47	9.4	8.4
		123	20	10	12	12	10	64	12.8	
44	Muck	124	10	8	10	12	10	50	10.0	
		125	6	6	5	9	0	26	5.2	7.1
		126	3	12	8	3	5	31	6.2	
45	Sandy soil	127	22	14	16	0	5	57	11.4	
		128	10	0	8	4	6	28	5.6	8.5
		129	....	....	....	....	....	.....	.....	
46	Controls	130	0	0	0	0	0	0	0	
		131	0	0	0	0	0	0	0	0.2
		132	0	3	0	0	0	3	0.6	
47	Controls	133	0	0	0	....	....	0	0	
		134	0	0	0	0	....	0	0	0
		135	0	0	0	0	0	0	0	

The average numbers of nodules per plant for all the plants inoculated with each of the given cultures appear in the last column in table 12. In table 13 these numbers are rearranged in numerical order according to the average number of nodules per plant produced by each culture:

TABLE 13. AVERAGE NUMBER OF NODULES PER PLANT IN SECOND TEST, ARRANGED IN NUMERICAL ORDER

Medium	Organisms living or not living*	Number of nodules per plant
Medium 334 + 1 per cent Witte's peptone	+	15.8
Medium 334 + 5 per cent gelatin	+	13.9
Medium 334 + 0.2 per cent calcium nitrate	+	13.1
Medium 334 + 2 per cent glycerin	+	12.1
Medium 334 + 0.5 per cent amygdalin	+	12.0
Medium 334 + 0.6 per cent potassium nitrate	+	10 6
Medium 334 + 10 per cent cane sugar	+	10 2
Cornstarch	+	9 9
Wheat middlings	+	9 8
Medium 334 + 2 per cent galactose	+	9.7
Medium 334 + 40 per cent cane sugar	+	9.2
Medium 334 + 0.1 per cent asparagin	+	8.8
Medium 334 + 2 per cent dextrose	+	8.7
Medium 334 + 2 per cent cane sugar	+	8.6
Wheat bran	+	8.6
Sandy soil	+	8 5
Compost	+	8.4
Fresh cow feces	+	8.4
Medium 334 + 2 per cent cane sugar	+	7.5
Partly decomposed cow feces	+	7.4
Muck	+	7.1
Canada field pea roots	+	7.0
Medium 334 + 0.1 per cent potassium nitrate	+	6.9
Medium 334 + 0.1 per cent calcium nitrate	+	6.7
Medium 334 + 1 per cent Merck's peptone	+	6 3
Medium 334 + 0.2 per cent potassium nitrate	+	5.3
Medium 334 + 0.6 per cent calcium nitrate	+	5.2
Medium 334 + 0.2 per cent Merck's peptone	+	4 4
Medium 334 + 20 per cent cane sugar	+	3.7
Medium 334 + 1 per cent salicin	0	3.0
Medium 334 + 0.5 per cent resorcin	0	2 6
Canada field pea hay	0	2.6
Medium 334 + 0.2 per cent phloroglucin	0	2.2
Medium 334 + 2 per cent lactose	+	2.1
Sawdust	+	1.6
Medium 334 + 2 per cent Witte's peptone	0	1.6
Soy bean roots	0	0.9
Medium 334 + 0.5 per cent potassium citrate	0	0.7
Medium 334 + 0.5 per cent potassium oxalate	0	0.6
Medium 334 + 5 per cent Witte's peptone	0	0.4
Controls (not inoculated)	0	0.2
Corn meal (culture contaminated)	0	0.1
Controls (not inoculated)	0	0
Soy bean hay	0	0

* + indicates living organisms; 0 indicates no living organisms.

In making any deductions from the preceding data, it must be remembered that the plants in this and in the other experiments were grown under special conditions. If the number of nodules on the plants in this experiment were dependent only on the degree of infecting power of the organism, an excellent illustration of variation in the infecting power would here be shown. This variation would, in this case, be due to the nature of the medium in which the organism was propagated.

That the number of nodules on a plant may be influenced by other factors than the infecting power of the organism has been shown in Part II of this paper, and also by experiments of other investigators. But the part that such other factors have played in this second test must be only a conjecture.

In order that a nodule may be produced, it is necessary that at least one organism shall come in contact with the root, that it shall enter the tissue of the root, and that it shall multiply inside the tissue. At least six factors can be mentioned which may have been of some importance in this experiment in bringing about this result:

1. *The distribution of the organisms through the entire volume of the soil in each flowerpot.*—The plant roots grow rapidly during the first three weeks after planting, and unless the organisms are evenly distributed through the soil a variation in the number of nodules might result if the plants are not allowed to grow for more than three weeks. Watering would tend to bring about an even distribution. All the flowerpots were watered twice each week, but the amount of water introduced into each pot was not measured — although it was uniformly constant — and because of this the distribution of the organisms throughout the soil may not have been uniform in all the flowerpots during the first two weeks.

2. *The number of organisms introduced into the different flowerpots at the time of inoculation.*—There is no available evidence to show how important this factor may be. That it may have exercised some influence seems highly probable. Reference to table 10 shows that there was a very great difference in the total number of organisms in the various cultures, and consequently the flowerpots inoculated with these cultures did not receive the same number of organisms.

3. *Multiplication of the organisms in the soil after inoculation.*—*Bacillus radicicola* multiplies readily in the sterilized soil that was used for growing the plants. This does influence the total number and the distribution

of the organisms in the soil. Moreover, if the infecting power of the organism were easily affected by the medium, it would be easily affected by the soil into which the organisms were introduced at the time of inoculation. The result would be that in course of time the infecting power of the different organisms, although different at the beginning, would become the same for all the organisms. If this is true, the difficulty in measuring the infecting power of a given culture is very evident.

4. *Resistance of the plant against the invasion of the organism.*—Nothing is known of this in connection with the leguminous plants and the nodule-forming organism. If such a character does exist in the plants, it probably varies in the different individuals and would influence the number of nodules formed.

5. *Infection from other sources than the inoculating material.*

6. *Infecting power of the organisms.*

It is evident from the above discussion that the number of nodules on plants may be the result of several factors operating simultaneously, and that it would be a difficult matter to determine their influence singly. The number of nodules, therefore, is not an accurate measure of the infecting power of the different cultures. Unfortunately, however, no other measure is available. In interpreting the above data, therefore, the limitations in the accuracy of the method must be borne in mind.

Comparing the data in table 13 with those in table 10, a close relation may be observed between the condition of the cultures when ten weeks old and the number of nodules on the plants inoculated with these cultures. The following cultures had no living organisms:

Medium 334 + 2 per cent Witte's peptone
Medium 334 + 5 per cent Witte's peptone
Medium 334 + 0.5 per cent resorcin
Medium 334 + 1 per cent salicin
Medium 334 + 0.5 per cent potassium oxalate
Medium 334 + 0.5 per cent potassium citrate
Medium 334 + 0.2 per cent phloroglucin (few organisms)
Soy bean hay
Soy bean roots
Canada field pea hay
Corn meal

Reference to table 13 shows that the plants inoculated with these cultures produced three nodules or less per plant. Whether the few nodules found

resulted from contamination or from a few surviving organisms not detected by the plating can only be conjectured. The remaining cultures all produced good inoculation, with the exception of the sawdust and the lactose culture, the average number of nodules per plant varying between 3.7 and 15.8. To what extent this variation in the number of nodules is due to the infecting power of the different cultures it is difficult to say with certainty; but that a part of the variation is due to other causes is evident from the data in the next to the last column in table 12. In this column the figures represent the average number of nodules per plant for every flowerpot. Since three flowerpots were inoculated with each culture, the extent of variation in the number of nodules in these pots must be due to other causes than the infecting power. A number of the more pronounced cases of variation in the number of nodules in the three respective flowerpots which were inoculated by the same culture are shown in table 14:

TABLE 14. VARIATION IN AVERAGE NUMBER OF NODULES IN SOME OF THE FLOWERPOTS

Inoculated with culture number	Average number of nodules in		
	Flowerpot 1	Flowerpot 2	Flowerpot 3
28	24.7	7.8	16.7
31	21.8	8.4	11.6
21	15.2	6.2	11.2
17	14.7	8.0	14.4
7	13.5	6.6	8.2
38	13.0	7.2	9.5
43	12.8	1.7	9.4
18	12.0	6.0	8.2
41	12.0	4.7	7.8
45	11.4	5.6	.....
1	11.0	6.2	.....
8	10.7	5.5	9.6
2	10.6	4.6	7.2
44	10.0	5.2	6.2
34	9.2	3.8	8.0
6	5.2	1.6	4.4

The three flowerpots inoculated with culture 43 had 12.8, 1.7, and 9.4 nodules per plant, respectively. A similar relation exists between the other flowerpots considered in table 14. If the efficiency of these cultures were based on the number of nodules per plant, culture 43, for example,

would be either very efficient or much reduced in infecting power, depending on which flowerpot was taken.

It seems apparent, from the preceding discussion, that it is extremely difficult to measure any variations in the infecting power of different cultures. When the great danger of infection of plants grown in sterile soil but not under sterile conditions is considered, and also the number of other factors that may affect nodule development on plants, not many clear-cut conclusions can be drawn from the second test. If the infecting power is measured by the number of nodules, by their size, by the uniformity of their distribution, and by their location, the following conclusions seem reasonable:

1. The cultures producing more than three nodules per plant are all efficient.

2. If one culture produced 3.7 nodules per plant and another culture produced 15.8 nodules per plant, the belief that the latter culture possesses greater infecting power than the former is not justified.

3. Some cultures produced no nodules, or only a few nodules confined to only one or two plants. Such cultures unquestionably lost their efficiency, but this loss of efficiency was parallel with the condition of the cultures. When no living organisms were found in a culture by using the plate method, such a culture produced no inoculation; and when living organisms were found, inoculation was produced.

4. Propagating and keeping *B. radicicola* of Canada field pea for ten weeks on media rich in nitrogenous matter, such as wheat bran, wheat middlings, fresh cow feces, and potassium and calcium nitrates, did not destroy the infecting power of the organism. If any injury to infecting power was caused by these substances, it could not be detected by the methods used in this experiment.

Third test of infecting power (table 15)

Cultures twenty weeks old were employed in this experiment. In inoculating the plants a known number of organisms was introduced into each flowerpot. By correlating the relation between the number of organisms used for the inoculation of each flowerpot and the number of nodules on the plants, it was hoped to find a more nearly exact measure of the infecting power of the organisms propagated in the different media. The results are summarized in table 15:

TABLE 15. INFECTING POWER OF CULTURES TWENTY WEEKS OLD

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Labcratory number of flowerpots	Number of nodules on each plant							Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture	Number of organisms introduced into each flowerpot
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	Plant 7				
1	Medium 334 + 2 per cent cane sugar....	1	10	6	5	2	8	....	....	31	6.2		324,000
		2	4	2	0	0	4	....	....	10	2.0	4.5	324,000
		3	8	14	0	0	....	....	....	22	5.5		324,000
3	Medium 334 + 2 per cent cane sugar	4	10	14	6	0	....	....	....	30	7.5		259,200
		5	6	1	0	0	....	....	....	7	1.7	4.8	259,200
		6	8	6	0	6	5	....	....	25	5.0		259,200
4	Medium 334 + 10 per cent cane sugar....	7	0	0	0	0	0	....	....	.....	.....		90,000
		8	0	0	0	0	0	5	....	5	8.3	3.1	90,000
		9	0	0	0	0	0	....	....	.....	.....		90,000
5	Medium 334 + 10 per cent cane sugar	10	0	5	4	6	....	....	....	15	3.7		5,000
		11	0	0	0	0	0	....	....	0	0	1.4	5,000
		12	0	0	3	0	3	0	....	6	1.0		5,000
6	Medium 334 + 20 per cent cane sugar	13	0	0	0	0	0	....	....	0	0		10
		14	0	0	0	6	7	....	....	13	2.6	3.0	10
		15	6	12	8	....	....	....	....	26	8.7		10
7	Medium 334 + 40 per cent cane sugar	16	0	0	0	0	0	0	0	0	0		0
		17	0	0	0	0	....	....	....	0	0	0	0
		18	0	0	0	....	....	....	....	0	0		0
8	Medium 334 + 2 per cent dextrose	19	8	4	4	0	....	....	....	16	4.0		52,000
		20	0	6	6	8	....	....	....	20	5.0	4.4	52,000
		21	4	....	....	....	....	....	....	4	4.0		52,000
9	Medium 334 + 2 per cent lactose	22	5	4	12	6	8	0	....	35	5.8		180
		23	8	6	0	0	4	0	3	21	3.0	5.3	180
		24	8	0	16	10	....	....	....	34	8.5		180
10	Medium 334 + 2 per cent galactose	25	12	10	6	16	....	....	....	44	11.0		60,000
		26	5	4	15	6	0	5	8	43	6.1	5.9	600
		27	0	0	0	3	4	7	....	14	2.3		600
11	Medium 334 + 2 per cent mannite	28	10	1	0	10	14	7	....	42	7.0		250,000
		29	12	5	2	8	2	....	....	29	5.8	7.6	2,500
		30	16	8	12	....	....	....	....	36	12.0		2,500
12	Medium 334 + 2 per cent glycerin	31	12	15	6	7	5	10	3	58	8.3		2,000,000
		32	8	14	15	15	16	10	8	86	12.3	10.5	20,000
		33	8	9	7	11	18	14	....	67	11.2		20,000
13	Medium 334 + 0.5 per cent potassium oxalate	34	0	0	0	0	0	....	....	0	0		0
		35	0	0	0	0	0	0	....	0	0	0.3	0
		36	0	0	0	5	....	....	....	5	1.2		0
14	Medium 334 + 0.2 per cent phloroglucin	37	0	0	0	0	0	0	....	0	0		0
		38	0	0	4	8	6	....	....	18	3.6	1.6	0
		39	....	....	....	....	....	....	....	.....	.....		0
15	Medium 334 + 0.5 per cent resorcin	40	0	0	0	0	....	....	....	0	0		0
		41	0	0	0	0	0	....	....	0	0	0	0
		42	0	0	0	0	0	....	....	0	0		0
16	Medium 334 + 1 per cent salicin	43	0	0	0	0	0	0	....	0	0		0
		44	0	....	....	....	....	....	....	0	0	0	0
		45	0	0	0	....	....	....	....	0	0		0

TABLE 15 (*continued*)

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Laboratory number of flowerpots	Number of nodules on each plant							Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture	Number of organisms introduced into each flowerpot
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	Plant 7				
17	Medium 334 + 0.5 per cent amygdalin	46	0	0	0	0	....	....	....	0	0		0
		47	0	0	0	0	0	....	....	0	0	0	0
		48	0	0	0	....	....	....	....	0	0		0
18	Medium 334 + 0.1 per cent asparagin	49	5	16	17	18	7	0	....	63	10.5		10,800,000
		50	6	15	12	10	6	....	....	49	9.8	10.0	108,000
		51	8	0	16	12	18	4	....	58	9.7		108,000
19	Medium 334 + 0.1 per cent potassium nitrate	52	25	17	6	0	....	....	....	48	12.0		7,200,000
		53	0	8	10	0	26	....	....	44	8.8	9.5	72,000
		54	20	12	10	4	4	0	....	50	8.3		72,000
20	Medium 334 + 0.2 per cent potassium nitrate	55	3	5	3	10	5	3	....	29	4.8		224,000
		56	5	5	0	0	....	....	....	10	2.5	3.9	224,000
		57	....	....	....	....	....	....	....	....	....		224,000
21	Medium 334 + 0.6 per cent potassium nitrate	58	0	10	12	4	14	0	....	40	6.7		14,400,000
		59	14	3	12	....	....	....	....	29	9.7	6.7	144,000
		60	6	10	0	2	....	....	....	18	4.5		144,000
22	Medium 334 + 0.1 per cent calcium nitrate	61	12	12	8	2	6	....	....	40	8.0		48,600,000
		62	2	1	7	9	5	3	....	27	4.5	6.7	486,000
		63	8	4	16	0	5	14	....	47	7.8		486,000
23	Medium 334 + 0.2 per cent calcium nitrate	64	2	1	8	14	12	16	....	53	8.8		10,800,000
		65	20	8	5	6	16	18	6	79	11.3	8.6	108,000
		66	5	3	6	14	3	8	1	40	5.7		108,000
24	Medium 334 + 0.6 per cent calcium nitrate	67	6	10	8	11	....	....	....	35	8.7		13,000,000
		68	3	7	8	9	11	7	8	53	7.6	9.2	130,000
		69	13	8	10	16	12	....	....	59	11.8		130,000
25	Medium 334 + 0.5 per cent potassium citrate	70	12	12	16	4	6	....	....	50	10.0		300,000
		71	18	8	4	20	....	....	....	50	12.5	9.1	3,000
		72	1	0	8	12	7	....	....	28	5.6		3,000
26	Medium 334 + 0.2 per cent Merck's peptone	73	1	0	1	12	10	0	....	24	4.0		800,000
		74	3	5	12	12	....	....	....	32	8.0	5.9	8,000
		75	8	0	0	14	6	10	....	38	6.3		8,000
27	Medium 334 + 1 per cent Merck's peptone	76	2	4	10	14	....	....	....	30	7.5		32,400,000
		77	10	13	15	18	4	....	....	59	11.8	9.5	324,000
		78	12	13	0	....	....	....	....	25	8.3		324,000
28	Medium 334 + 1 per cent Witte's peptone	79	3	7	3	0	5	....	....	18	3.6		21,600,000
		80	4	12	10	12	14	15	20	87	12.4	9.4	216,000
		81	12	8	16	....	....	....	....	36	12.0		216,000
29	Medium 334 + 2 per cent Witte's peptone	82	12	4	4	10	....	....	....	30	7.5		64,800,000
		83	4	0	12	8	....	....	....	24	6.0	7.9	648,000
		84	10	14	9	....	....	....	....	33	11.0		648,000
30	Medium 334 + 5 per cent Witte's peptone	85	0	0	0	2	0	0	....	2	0.3		0
		86	0	0	0	0	0	0	....	0	0	0.1	0
		87	0	0	0	0	0	0	0	0	0		0
31	Medium 334 + 5 per cent gelatin	88	3	2	2	10	7	6	....	30	5.0		6,480,000
		89	5	8	7	6	8	2	....	36	6.0	6.6	64,800
		90	7	8	12	14	5	....	....	46	9.2		64,800

TABLE 15 (*concluded*)

Laboratory number of culture	Medium in which *B. radicicola* was propagated	Laboratory number of flowerpots	Number of nodules on each plant							Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture	Number of organisms introduced into each flowerpot
			Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	Plant 7				
32	Soy bean roots, ground	91	0	0	0	0	....	....	....	0	0		0
		92	0	0	0	0	....	....	....	0	0	0	0
		93	0	0	0	0	0	0	....	0	0		0
33	Soy bean hay, ground	94	0	0	0	0	0	....	....	0	0		0
		95	0	0	0	0	....	....	....	0	0	0	0
		96	0	0	0	0	0	0	....	0	0		0
34	Canada field pea roots, ground	97	0	0	0	0	0	0	0	0	0		0
		98	0	0	0	0	....	....	....	0	0	0	0
		99	0	0	0	0	....	....	....	0	0		0
35	Canada field pea hay, ground	100	0	0	0	....	....	....	....	0	0		0
		101	0	0	0	0	....	....	....	0	0	0	0
		102	0	0	0	0	0	0	....	0	0		0
36	Sawdust	103	15	....	....	....	....	....	....	15	15.0		350,000
		104	4	9	5	5	3	....	....	26	5.2	6.6	3,500
		105	5	....	....	....	....	....	....	5	5.0		3,500
37	Corn meal	106	6	10	8	16	....	....	....	40	10.0		9,700,000
		107	1	2	3	12	5	14	....	37	6.2	7.5	97,000
		108	12	6	8	8	1	....	....	35	7.0		97,000
38	Cornstarch	109	12	7	14	....	....	....	....	33	11.0		9,700,000
		110	5	6	....	....	....	....	....	11	5.5	9.1	97,000
		111	8	7	14	....	....	....	....	29	9.7		97,000
39	Wheat bran	112	7	12	10	....	....	....	....	29	9.7		60,000,000
		113	1	2	22	8	....	....	....	33	8.2	9.8	600,000
		114	14	22	0	....	....	....	....	36	12.0		600,000
40	Wheat middlings	115	20	10	2	10	....	....	....	42	10.5		42,000,000
		116	15	12	16	....	....	....	....	43	14.3	12.0	420,000
		117	12	10	13	....	....	....	....	35	11.7		420,000
41	Compost	118	12	14	8	0	....	....	....	34	8.5		12,000,000
		119	8	2	6	....	....	....	....	16	5.3	7.3	120,000
		120	9	4	10	....	....	....	....	23	7.7		120,000
42	Partly decomposed cow feces	121	5	4	5	....	....	....	....	14	4.7		324,000,000
		122	6	12	7	3	16	20	....	64	10.7	8.3	3,240,000
		123	6	0	18	12	2	....	....	38	7.6		3,240,000
43	Fresh cow feces	124	10	12	7	15	....	....	....	44	11.0		81,000,000
		125	14	0	5	12	....	....	....	31	7.7	9.4	810,000
		126	....	....	....	....	....	....	....	....	....		810,000
44	Muck	127	4	0	0	0	....	....	....	4	1.0		24,000,000
		128	8	12	0	0	....	....	....	20	5.0	3.5	240,000
		129	1	8	7	2	....	....	....	18	4.5		240,000
45	Sandy soil	130	21	10	7	6	....	....	....	44	11.0		2,000,000
		131	17	14	16	2	....	....	....	49	12.2	12.3	20,000
		132	12	22	8	....	....	....	....	42	14.0		20,000

In this test a known number of organisms was introduced into each flowerpot at the time of inoculation, in order to trace the relation between the number of nodules developed and the number of organisms used for inoculation. It was hoped that in this way some means might be found of measuring not only the loss, but also the degree, of infecting power. From the data in table 15 it is seen that absolutely no relation exists between the number of organisms introduced into the flowerpots and the number of nodules that developed, so that the only measure of infecting power is the presence or the absence of nodules. This, however, does not measure the degree of infecting power, but only its presence or absence.

The average number of nodules per plant produced by each culture, as shown in table 15, are rearranged in numerical order in table 16. It is found on comparing tables 13 and 16 that the results of the third test of infecting power show a general agreement with the results of the second test.

If it is considered that the presence of three or more nodules per plant indicates that the culture was efficient, the following cultures are seen to have lost their efficiency:

Soy bean hay
Soy bean roots
Canada field pea hay
Canada field pea roots
Medium 334 + 10 per cent cane sugar
Medium 334 + 40 per cent cane sugar
Medium 334 + 0.2 per cent phloroglucin
Medium 334 + 1 per cent salicin
Medium 334 + 0.5 per cent amygdalin
Medium 334 + 0.5 per cent resorcin
Medium 334 + 0.5 per cent potassium oxalate
Medium 334 + 5 per cent Witte's peptone

The cultures that had any living organisms at the time of inoculation produced nodules practically in all cases in the three tests. The cultures in which no living organisms were found produced no nodules, or only a few unevenly distributed. It must be remembered that a certain amount of contamination may occur, and that the method of determining the presence of living organisms by plate cultures is not absolutely accurate. The cultures in this test were ten weeks older than those in the second test. The results show that the nodule-bacteria cultures can be kept for at least twenty weeks, and the bacteria will still be efficient in producing nodules.

TABLE 16. AVERAGE NUMBER OF NODULES PER PLANT IN THIRD TEST, ARRANGED IN NUMERICAL ORDER

Medium	Organisms living or not living*	Number of nodules per plant
Sandy soil	+	12.3
Wheat middlings	+	12.0
Medium 334 + 2 per cent glycerin	+	10.5
Medium 334 + 0.1 per cent asparagin	+	10.0
Wheat bran	+	9.8
Medium 334 + 1 per cent Merck's peptone	+	9.5
Medium 334 + 0.1 per cent potassium nitrate	+	9.5
Medium 334 + 1 per cent Witte's peptone	+	9.4
Fresh cow feces	+	9.4
Medium 334 + 0.6 per cent calcium nitrate	+	9.2
Cornstarch	+	9.1
Medium 334 + 0.5 per cent potassium citrate	+	9.1
Medium 334 + 0.2 per cent calcium nitrate	+	8.6
Partly decomposed cow feces	+	8.3
Medium 334 + 2 per cent Witte's peptone	+	7.9
Medium 334 + 2 per cent mannite	+	7.6
Corn meal	+	7.5
Compost	+	7.3
Medium 334 + 0.1 per cent calcium nitrate	+	6.7
Medium 334 + 0.6 per cent potassium nitrate	+	6.7
Medium 334 + 5 per cent gelatin	+	6.6
Sawdust	+	6.6
Medium 334 + 2 per cent galactose	+	5.9
Medium 334 + 0.2 per cent Merck's peptone	+	5.9
Medium 334 + 2 per cent lactose	+	5.3
Medium 334 + 2 per cent cane sugar	+	4.8
Medium 334 + 2 per cent cane sugar	+	4.5
Medium 334 + 2 per cent dextrose	+	4.4
Medium 334 + 0.2 per cent potassium nitrate	+	3.9
Muck	+	3.5
Medium 334 + 10 per cent cane sugar	+	3.1
Medium 334 + 20 per cent cane sugar	+	3.0
Medium 334 + 0.2 per cent phloroglucin	0	1.6
Medium 334 + 10 per cent cane sugar	+	1.4
Medium 334 + 0.5 per cent potassium oxalate	0	0.3
Medium 334 + 5 per cent Witte's peptone	0	0.1
Medium 334 + 40 per cent cane sugar	0	0
Medium 334 + 0.5 per cent resorcin	0	0
Medium 334 + 1 per cent salicin	0	0
Medium 334 + 0.5 per cent amygdalin	0	0
Canada field pea hay	0	0
Canada field pea roots	0	0
Soy bean hay	0	0
Soy bean roots	0	0

* + indicates living organisms; 0 indicates no living organisms.

EXPERIMENT 13

INFLUENCE OF MEDIA 300, 310, 335, AND 400

The organism used for this experiment was propagated on medium 335, and had been kept in the laboratory for two years and three months, where it was exposed to diffused light and transfers had been made at intervals. When the experiment was started the organism readily produced nodules on the plants, showing that its infecting power had not been lost.

The plan of the experiment was to propagate the organism on both the nitrogen-free and the nitrogenous media for a period of time, and then to test these cultures for nodule production. The following procedure was adopted: Media 300, 310, 335, and 400 were introduced into test tubes, sterilized, and sloped. Four slopes from each of these media were inoculated with the same culture of the organism. A few days later a second set of four slopes from each medium was inoculated, and so on. Nine such sets of agar slopes from each of the four media were inoculated in one hundred and fifteen days. The four agar slopes of the same medium in each set were inoculated from one of the four slopes of the same medium from the previous set, so that on all the slopes of the same medium in the nine sets the organism was under the influence of the same media for one hundred and fifteen days. The age of the agar slopes, however, differed in the respective sets. When the ninth set was inoculated, four additional tubes of media 300, 310, and 400 were inoculated with a culture of the organism which up to that time had been propagated on nitrogen-free medium 335. These test-tube cultures are designated in table 17 as set X. They were fifteen days old and had been under the influence of nitrogenous media for only fifteen days when tested for infecting power. In order to prevent the effects of drying, melted paraffin was poured on the cotton plugs after a good growth had developed. All the cultures were kept at room temperature.

The infecting power of these agar-slope cultures was tested by inoculating Canada field peas. The method of growing and examining the plants was the same as in experiment 12. In inoculating the plants, the growth from each agar slope was introduced into 100 cubic centimeters of sterile water, the number of organisms in this was determined by the plate method, and a definite quantity of this infusion was poured over the seeds in each flowerpot.

Unfortunately, an accident happened to a large number of the flowerpots in the greenhouse, and consequently the data are not complete. As far as they could be obtained, they are summarized in table 17:

TABLE 17. INFLUENCE OF MEDIA 300, 310, 335, AND 400

Laboratory number of culture	Number of set	Medium	Age (in days) of culture when used for inoculation	Laboratory number of flowerpots	Number of nodules on each plant						Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture	Number of living organisms found in the culture
					Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6				
1	I	335	115	1	0	6	14	7	6	6	39	6.5	9.8	300,000,000
				2	13	15	12	7	8	....	55	11.0		
				3	25	16	2	....	....	....	43	14.3		
2	I	300	115	4	0	0	0	0	0	0	0	0	0	0
				5	0	0	0	0	....	....	0	0		
				6	0	0	0	....	....	....	0	0		
3	I	400	115	7	0	0	0	0	0	0	0	0	0	0
				8	0	0	0	0	0	0	0	0		
				9	0	0	0	0	0	0	0	0		
4	II	335	110	10	12	13	8	8	5	....	46	9.2	7.3	350,000,000
				11	6	8	8	5	5	2	34	5.7		
				12	....	....	....	....	....	....	....	....		
5	II	300	110	13	0	0	0	0	0	0	0	0	0	0
				14	0	0	0	0	0	0	0	0		
				15	0	0	0	0	....	....	0	0		
6	II	400	110	16	0	0	0	0	0	....	0	0	0.6	0
				17	0	0	0	0	0	4	4	0.7		
				18	0	0	0	0	6	....	6	1.2		
7	III	335	105	19	8	9	12	20	14	....	63	12.6	9.5	360,000,000
				20	9	20	6	7	4	....	46	9.2		
				21	8	10	7	8	0	....	33	6.6		
8	III	300	105	22	0	0	0	0	0	0	0	0	0	0
				23	0	0	0	0	0	0	0	0		
				24	0	0	0	0	0	0	0	0		
9	III	400	105	25	0	0	0	0	0	0	0	0	0	0
				26	0	0	0	0	0	0	0	0		
				27	0	0	0	0	0	0	0	0		
10	III	310	105	28	12	5	15	4	10	15	61	10.2	10.7	144,000,000
				29	14	16	6	18	8	13	75	12.5		
				30	7	10	6	12	....	....	35	8.7		
11	IV	400	98	31	0	2	0	0	....	....	2	0.5	0.5	90,000
				32	....	....	....	....	....	....	....	....		
				33	....	....	....	....	....	....	....	....		
12	VI	335	81	34	13	1	4	17	6	....	41	8.2	6.9	180,000,000
				35	4	2	14	8	2	5	35	5.8		
				36	14	10	6	0	8	3	41	6.8		
13	VI	300	81	37	0	0	0	0	0	0	0	0	0	0
				38	....	....	....	....	....	....	....	....		
				39	....	....	....	....	....	....	....	....		
14	VI	400	81	40	10	11	12	7	0	....	40	8.0	8.5	1,000,000
				41	0	20	6	7	13	8	54	9.0		
				42	12	10	6	6	....	....	34	8.5		
15	VI	310	81	43	20	15	8	0	....	....	43	10.7	10.7	540,000,000
				44	....	....	....	....	....	....	....	....		
				45	....	....	....	....	....	....	....	....		

TABLE 17 (*concluded*)

Laboratory number of culture	Number of set	Medium	Age (in days) of culture when used for inoculation	Laboratory number of flowerpots	Number of nodules on each plant						Total number of nodules in each flowerpot	Average number of nodules per plant in each flowerpot	Average number of nodules per plant produced by the culture	Number of living organisms found in the culture
					Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6				
16	VII	355	61	46	12	0	6	8	0	16	42	7.0	9.8	396,000,000
				47	16	7	15	6	20	....	64	12.8		
				48	3	20	0	6	22	9	60	10.0		
17	VII	400	61	49	2	10	4	....	....	....	16	5.3	7.7	10,000,000
				50	5	5	12	16	....	....	38	9.5		
				51	....	....	....	....	....	....	....	....		
18	VII	310	61	52	0	0	0	0	0	0	0	0	0	0
				53	0	0	0	0	0	0	0	0		
				54	0	0	0	0	0	0	0	0		
19	VIII	335	49	55	0	10	8	9	0	....	27	5.4	6.2	216,000,000
				56	8	5	0	....	....	....	13	4.3		
				57	8	10	9	7	....	....	34	8.5		
20	VIII	300	49	58	7	9	....	....	....	....	16	8.0	11.2	16,000,000
				59	6	7	18	7	16	12	66	11.0		
				60	6	13	30	4	....	....	53	13.2		
21	VIII	400	49	61	8	0	2	18	0	....	28	5.6	6.8	252,000,000
				62	12	13	4	4	14	....	47	9.4		
				63	4	0	1	6	16	....	27	5.4		
22	VIII	310	49	64	0	6	25	....	....	....	31	10.3	7.8	8,000,000
				65	6	10	6	5	....	....	27	6.7		
				66	12	2	0	10	12	....	36	7.2		
23	IX	335	15	67	10	6	5	2	8	....	31	6.2	4.5	576,000,000
				68	4	2	0	0	4	....	10	2.0		
				69	8	14	0	0	....	....	22	5.5		
24	IX	300	15	70	12	35	23	0	....	....	70	15.0	8.5	4,320,000,000
				71	18	0	0	13	5	8	44	7.3		
				72	0	7	0	2	5	....	14	2.8		
25	IX	400	15	73	0	5	8	0	....	....	13	3.2	5.8	2,592,000,000
				74	10	4	4	2	7	10	37	6.2		
				75	7	18	0	....	....	....	25	8.3		
26	IX	310	15	76	9	5	4	7	0	0	25	4.2	6.2	3,240,000,000
				77	8	0	4	7	....	....	19	4.7		
				78	12	8	8	15	0	12	55	9.2		
27	X	300	15	79	5	0	0	12	6	....	23	4.6	7.1	180,000,000
				80	4	7	30	20	12	....	73	14.6		
				81	0	0	3	3	5	....	11	2.2		
28	X	400	15	82	2	15	2	8	6	....	33	6.6	7.1	972,000,000
				83	5	9	1	6	6	8	35	5.8		
				84	6	8	0	25	6	8	53	8.8		
29	X	310	15	85	6	0	0	7	7	4	24	4.0	5.2	1,152,000,000
				86	0	0	0	0	7	10	17	2.8		
				87	12	25	0	....	....	....	37	12.3		

Results

The nature and the composition of the four media used in experiment 13 are given in table 1, page 11. No nitrogen in any form was added to medium 335. The other three media received 0.3 per cent of Liebig's beef extract, and, in addition to this, media 300 and 310 received 1 per cent and medium 400 received 2 per cent of Witte's peptone. Medium 335 contained 2 per cent of cane sugar, and medium 310 contained 2 per cent of dextrose.

As seen from table 17, the age of the individual cultures varied between fifteen days and one hundred and fifteen days; but the organisms in all the cultures, except 27, 28, and 29, had been under the influence of their respective media for one hundred and fifteen days. Cultures 27, 28, and 29 were fifteen days old and had been under the influence of their respective media for only fifteen days, having been grown previously on medium 335.

The influence of these four media on the organism is shown in table 17. In order to bring out the relations more clearly, a part of these data are rearranged in table 18:

TABLE 18. INFLUENCE OF AGE OF CULTURES ON THE GIVEN MEDIA

Laboratory number of culture	Culture medium	Age of culture (days)	Average number of nodules per plant	Number of living organisms in culture
23	335	15	4.5	576,000,000
19	335	49	6.2	216,000,000
16	335	61	9.8	396,000,000
12	335	81	6.9	186,000,000
7	335	105	9.5	360,000,000
4	335	110	7.3	350,000,000
1	335	115	9.8	300,000,000
24	300	15	8.5	4,320,000,000
20	300	49	11.2	16,000,000
13	300	81	0	0
8	300	105	0	0
5	300	110	0	0
2	300	115	0	0
25	400	15	5.8	2,592,000,000
21	400	49	6.8	252,000,000
17	400	61	7.7	10,000,000
14	400	81	8.5	1,000,000

TABLE 18 *(concluded)*

Laboratory number of culture	Culture medium	Age of culture (days)	Average number of nodules per plant	Number of living organisms in culture
11	400	98	0.5	90,000
9	400	105	0	0
6	400	110	0.6	0
3	400	115	0	0
26	310	15	6.2	3,240,000,000
22	310	49	7.8	8,000,000
18	310	61	0	0
15	310	81	10.7	540,000,000
10	310	105	10.7	144,000,000
27	300	15	7.1	180,000,000
28	400	15	7.1	972,000,000
29	310	15	5.2	1,152,000,000

The organism had been propagated and kept on medium 335, a non-nitrogenous medium, for over two years before this experiment was started. Several tests of infecting power were made during that time. This medium was used also in experiment 12. As far as could be judged by all these tests, the infecting power of the organism remained constant when propagated and kept on this medium. Therefore the results shown in experiment 13 as to the infecting power of the cultures on medium 335 are taken to represent the normal infecting power of the organism, and serve as a basis for comparison of the infecting power of the cultures on the other media.

Of the seven cultures on medium 300, a nitrogenous medium, three cultures — namely, 20, 24, and 27 — produced nodules. Cultures 24 and 27 were fifteen days old and culture 20 was forty-nine days old. The organisms in cultures 20 and 24 were under the influence of medium 300 for one hundred and fifteen days, and culture 27 for only fifteen days. These three cultures appeared as effective as those on medium 335. The remaining cultures on medium 300 were from eighty-one to one hundred and fifteen days old and did not produce any nodules, apparently having lost their power of infection.

Similar results were obtained with the nitrogenous medium 400. Of

the nine cultures on this medium, five produced nodules, two were doubtful, and two produced no nodules. Culture 28 was fifteen days old and had been under the influence of this medium for only fifteen days; while culture 25 was fifteen days old and had been under the influence of this medium for one hundred and fifteen days. No appreciable difference in the infecting power of these two cultures was observed.

Medium 310 is the same as medium 300 except that it contains 2 per cent of dextrose. Five of the six cultures on this medium produced nodules; one did not, and that was not the oldest culture. Comparing these results with those from medium 300, it seems as if the dextrose protected the infecting power of the organism on this medium.

In the last column of Table 18 the number of living organisms in each culture is given, and in the next to the last column the number of nodules per plant produced by each culture is given. It is seen that in all cases in which no living organisms were found in a culture no nodules were produced by that culture, and that the cultures in which living organisms were present produced nodules. Culture 6 is the only exception, but in this case only two plants out of sixteen had four and six nodules, respectively, and the size and location of these nodules pointed to later contamination.

According to the above data, *Bacillus radicicola* of Canada field pea does not lose its infecting power when propagated on medium 300 and 400 for one hundred and fifteen days. Some of the cultures on these two media did lose their infecting power, but in all cases these were the older cultures. This loss in efficiency is due to death of the culture, and death is induced by nitrogenous media after a considerable duration.

SUMMARY

1. The causal organism in the case of Canada field pea nodules is *Bacillus radicicola*. Its flagella are peritrichic, and eight was the largest number found. Its group number is B. 222.2322033.

2. Nodules developed both in light and in darkness. A larger number of nodules, however, developed in darkness.

3. Nodules developed readily both in the soil extract and in synthetic nutrient solutions in which the nitrates were either omitted or replaced by chlorides. The nodules continued to increase in number as long as the plants continued to grow.

4. In a full nutrient solution containing nitrates a few nodules may develop immediately after inoculation, but a subsequent continual development of nodules seems to be inhibited.

5. No nodule development took place in nutrient solutions in which the individual essential elements were omitted, except in the case of nitrogen.

6. In sandy soil a moisture content of 20 to 40 per cent was more favorable for nodule development than lower or higher percentages.

7. The addition of KNO_3, $Ca(NO_3)_2$, NH_4Cl, $FeCl_3$ KCl, or peptone to sandy soil in the proportion of $\frac{1}{4}$ gram of the salts to 300 grams of the soil, air-dry, had an inhibiting effect on nodule development on Canada field peas. The addition of $MgSO_4$, KH_2PO_4, $Ca(H_2PO_4)_2$, and tannic acid, especially at the lower concentrations, in 300 grams of the soil, had a beneficial effect on nodule development on Canada field peas.

8. Nutrition markedly influences the morphology of the nodule organisms.

9. The addition of 1 cubic centimeter or more of a normal solution of HCl to 10 cubic centimeters of agar medium 334, 335, or 337 was injurious to the vitality, and therefore to the infecting power, of the alfalfa-nodule organism. The addition of 2 cubic centimeters or less of a normal solution of NaOH to 10 cubic centimeters of each of the above media seemed to be slightly beneficial to the vitality and the infecting power of this organism.

10. The organism of Canada field peas produced no visible growth in medium 334 when the following substances were added: levulose 2 per cent, phloroglucin 0.2 per cent, potassium oxalate 0.5 per cent, potassium citrate 0.5 per cent, Witte's peptone 5 per cent, Merck's peptone 3 per cent.

11. The organism multiplies readily in some soils and in various substances; as many as 10,000,000,000 organisms per gram developed in wheat bran and in ground peas.

12. The infecting power of *B. radicicola* of Canada field pea was not affected after the organism had been kept on medium 335 for two years and a half in the laboratory, the culture being transferred once each month.

13. The infecting power of the organism was not appreciably influenced by the various media. All the cultures in which living nodule-forming organisms were found at the time of trial produced nodules.

14. In some media and under certain conditions the organisms died much sooner than in other media. The nitrogenous media did not seem to influence the infecting power of the organism.

15. It is not difficult to determine whether or not a given culture can produce nodules, but there is no accurate method of measuring the slight variations in infecting power that may exist in the different cultures.

GENERAL DISCUSSION

The definition of *virulence* as given by various authors in the medical texts is not clear, but in general the word is defined as meaning the power of microorganisms to invade and multiply in the tissues of a host and cause some injury or disease to the host. It was found by Peirce (1902), Fred (1911), and others, that the cells in the nodule are injured by the nodule-forming organism and become abnormal, and that for this reason the relation between the microorganism and the legume is a parasitic one. Furthermore, a nodule on a leguminous plant is a swelling, a hypertrophic formation; and morphologically speaking, it is an abnormality, or a form of disease. Nodule formation, therefore, may be considered as of a pathological nature. But, it may be asked, which is the normal root of the legume — the one with the nodules, or the one without the nodules, or both? Whatever the morphological and cytological evidence may be, one well-established fact stands out; namely, that leguminous plants are benefited by the presence of nodules. No positive evidence has been produced thus far to show that the microorganism which causes nodules is injurious to leguminous plants. The nodule-forming organism penetrates the root tissue, multiplies therein, and apparently derives its necessary food therefrom; and in return for this it enables the plant to obtain a certain amount of nitrogen. There is, therefore, a mutual and beneficial exchange and the relation is symbiotic.

The primary object of the experiments reported in this investigation was to determine whether the power of the nodule-forming organism to cause nodules is easily altered by artificial media. The nodule-forming organism of Canada field pea was chosen for this purpose, and the results apply only to that organism. It is probable that the organisms from other legumes might have given different results. Since the value of the investigation depended so much on the securing of a pure culture, the various precautions, as previously indicated, were adopted for this purpose.

The fact that a pure culture is obtained from a nodule and resembles in its cultural characteristics the true nodule-forming organism is not a sufficient proof that such an organism is the nodule-forming organism. De' Rossi (1907), in his investigation, emphasizes this point, and unquestionably the information on the general subject of nitrogen fixation by leguminous plants has been colored by results from experiments in which some organism other than the nodule-forming organism was employed.

As stated on page 59, the number, size, and location of the nodules on the roots are probably influenced by a number of factors. Since the number of nodules produced on the plants in a given time was used as the measure of the infecting power of the culture with which the plants were inoculated, it seemed advisable to study the influence of several factors and thus to determine whether Canada field peas readily form nodules under the conditions that it was planned to use in Part III. The results of these experiments in Part II tend to point to the conclusion that, in general, the conditions favoring the normal development of plants favor also the development of nodules. An exception to this is found in the fact that the presence of nitrates tends to inhibit the development of nodules, and at the same time favor the normal development of the plants. No satisfactory explanation for this phenomenon has as yet been given. The plants appear not to be injured by the presence of nitrates, and neither do the nodule-forming organisms seem to be injured when propagated on a medium in which nitrate is present (see experiment 13). The explanation that the plants are made more vigorous when supplied with nitrogen, and can more readily resist the invasion of the microorganisms, cannot be taken seriously. It might be noted here that the root system of Canada field peas grown in Pfeffer's nutrient solution appears normal, tending to become slightly brownish; but when the nitrate in the same solution is replaced by the chloride of the same metal, the root system becomes larger, the roots being more numerous and longer. Consequently, the rate of growth of the root tissue is accelerated by the absence of nitrates. Whether this somewhat rapid growth of the root tissue has any relation to nodule formation is not known. It is highly probable that a biological factor also influences the development of nodules. The microbial flora of the soil or of the solution in which the plants are grown is undoubtedly influenced by the composition of that soil or solution. The microorganisms that thrive best in a highly nitrogenous soil or solu-

tion, and the products of their metabolic activities, may exercise an injurious influence on the nodule organisms and thus prevent them from multiplication and distribution through the soil.

The influence of artificial media on the power of *Bacillus radicicola* to cause nodules is of considerable importance, in view of the fact that inoculation for leguminous crops with pure cultures is extensively practiced and the pure cultures have to be propagated on some media. Frank (1899), in commenting on the low efficiency of nitragin, suggested that probably the medium (gelatin) on which the cultures were propagated and kept was not favorable for the organisms, and that this might be the cause of the low efficiency of nitragin. Hiltner (1900) finally substituted liquid media for the gelatin, and was able to obtain better results from the legume inoculation. Süchting (1904) found that not only the gelatin media are injurious to the bacteria — a point shown by Hiltner — but the agar media also may be unfavorable. Moore (1905) made the observation that the nodule-forming bacteria increase most rapidly on a medium rich in nitrogen, but that the resulting growth is usually very much reduced in infecting power. Lewis and Nicholson (1905), on the other hand, state that the presence or absence of nitrogen in the culture media is not the determining factor in maintaining the activity of the germ.

A reasonable conclusion from the investigations mentioned above seems to be that some artificial media are more favorable than others for the propagation of *B. radicicola*, and that the amount of growth is not always directly proportional to the nodule-producing efficiency of the organism. With this conclusion the experiments in Part III are in accord. In addition to this, the experiments point to the conclusion that *B. radicicola* of Canada field pea does not possess "virulence" in a pathological meaning of the word. The ability to cause nodules is so closely bound up with the general vitality of the bacteria that our means and methods cannot detect any variations, if such there are, in their nodule-producing ability. The writer's opinion is that every living nodule-producing organism in a vigorous condition, will, if given a chance, cause nodule development no matter on what kind of media it has been propagated. The propagation of the organisms on different media does not measurably affect their nodule-producing efficiency. The organisms die sooner on some media than on others, and the loss of the nodule-

producing efficiency of the cultures is due to the dying-out of the organisms. Whether the injurious agent is some ingredient of the media, or lack of a proper nutrient, or the accumulation of the metabolic products, has not been determined. The observation that a cop ous growth is usually of reduced nodule-producing efficiency suggests the last as the probable, or at least a partial, explanation.

When one examines leguminous plants grown under favorable conditions, one finds that each plant has a limited number of nodules and that the number and the size of the nodules vary on the different plants. Hiltner (1900), holding the relation between *B. radicicola* and the leguminous plants to be of a pathological nature, concluded from his experiments that the variableness of the infecting power of the nodule-forming bacteria is the limiting factor which determines the number and size of the nodules under otherwise favorable conditions. Süchting (1904), in trying to account for the limited number of nodules on each plant, emphasized the resistance of the plants against the invasion of the bacteria. His explanation is as follows: " In contrast to Hiltner's immunity theory, I am of the opinion that the nodule formation and their number are regulated by the relation between the antibodies in the plant and the infecting substance of the bacteria." [7]

These explanations do not appear to the writer to explain the conditions. No pathological explanation can account for a limited number of nodules. The physical condition and the chemical composition of the soil, the amount of moisture, and the microbial flora of the soil, are some of the important factors that interfere, directly or indirectly, with the coming together of *B. radicicola* and the roots of the plants. The stage in the development of the plant, the rate of growth of the roots, the number of root hairs, the character of the root tissue, and other factors, may also play a part in limiting the development of nodules.

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[7] Translation from the original German.

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